

MECHANISMS OF TRANSLATIONAL REGULATION IN THE PANCREATIC
 β CELL STRESS RESPONSE

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DEDICATION

I dedicate this to my family, without whose loving support, motivation, and friendship this work would not have been possible.

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The islet β cell is unique in its ability to synthesize and secrete insulin for use in the body. A number of factors including proinflammatory cytokines, free fatty acids, and islet amyloid are known to cause β cell stress. These factors lead to lipotoxic, inflammatory, and ER stress in the β cell, contributing to β cell dysfunction and death, and diabetes. While transcriptional responses to β cell stress are well appreciated, relatively little is known regarding translational responses in the stressed β cell. To study translation, I established conditions in vitro with MIN6 cells and mouse islets that mimicked UPR conditions seen in diabetes. Cell extracts were then subjected to polyribosome profiling to monitor changes to mRNA occupancy by ribosomes. Chronic exposure of β cells to proinflammatory cytokines (IL-1β, TNF-α, IFN-γ), or to the saturated free fatty acid palmitate, led to changes in global β cell translation consistent with attenuation of translation initiation, which is a hallmark of ER stress. In addition to changes in global translation, I observed transcript specific regulation of ribosomal occupancy in β cells. Similar to other privileged mRNAs (*Atf4*, *Chop*), *Pdx1* mRNA remained partitioned in actively translating polyribosomes during the UPR, whereas the mRNA encoding a proinsulin processing enzyme (*Cpe*) partitioned into inactively translating monoribosomes. Bicistronic luciferase reporter analyses revealed that the distal portion of the 5' untranslated region of

mouse *Pdx1* (between bp –105 to –280) contained elements that promoted translation under both normal and UPR conditions. In contrast to regulation of translation initiation, deoxyhypusine synthase (DHS) and eukaryotic translation initiation factor 5A (eIF5A) are required for efficient translation elongation of specific stress relevant messages in the β cell including *Nos2*. Further, p38 signaling appears to promote translational elongation via DHS in the islet β cell. Together, these data represent new insights into stress induced translational regulation in the β cell. Mechanisms of differential mRNA translation in response to β cell stress may play a key role in maintenance of islet β cell function in the setting of diabetes.

Raghavendra G Mirmira, MD, PhD, Chair

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ABBREVIATIONS

ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
ATP	Adenosine triphosphate
CHOP	C/EBP homologous protein
CPE	Carboxypeptidase E
DHS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
DM	Diabetes mellitus
eIF2 α	eukaryotic translation initiation factor 2 α
eIF5A	eukaryotic translation initiation factor 5 A
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
FFA	Free fatty acid
GC7	<i>N</i> ¹ -guanyl-1,7-diaminoheptane
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GTT	Glucose tolerance test
GSIS	Glucose stimulated insulin secretion
IAPP	Islet amyloid polypeptide
IFN- γ	Interferon γ
IL-1 β	Interleukin 1 β

IRE1	Inositol requiring enzyme 1
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factors
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MODY	Maturity onset diabetes of the youth
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Non obese diabetic
ORF	Open reading frame
PBS	Phosphate buffered saline
Pdx1	Pancreatic and duodenal homeobox 1
PERK	PRKR-like endoplasmic reticulum kinase
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RT	Room temperature
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase
STAT	Signal transducers and activators of transcription
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor α
UPR	Unfolded protein response

UTR Untranslated region

XPB1 X-box binding protein 1

CHAPTER 1

Introduction

1.1 THE ISLET β CELL

The pancreatic islet β cell is unique in its ability to synthesize and secrete insulin. Insulin is a highly conserved 51 amino acid peptide hormone that is required for the cellular uptake of glucose into adipose and muscle tissue, and for the suppression of glucose output by the liver. Insulin is synthesized by the β cell as a preprohormone, which is subsequently proteolytically processed in the endoplasmic reticulum (ER), Golgi, and secretory granules to the mature peptide, consisting of two polypeptide chains (1). Systemic resistance to the action of insulin, as well as defects in the secretion of insulin by islet β cells are risk factors for the development of diabetes (Fig. 1). Failure of the β cell to produce sufficient insulin to meet demand is a common feature of both type 1 and type 2 diabetes, and is the most important determinant of progression from the pre-diabetic state to frank diabetes (2). As such, research aimed at preserving β cell mass and function may offer new therapies for treatment of diabetes.

A. Mechanisms of Glucose Stimulated Insulin Secretion

β cell insulin secretion is carried out by mechanisms unique to the cell type. Insulin secretion requires a stimulus-secretion coupling mechanism known as glucose-stimulated insulin secretion (GSIS). When glucose concentrations in the blood rise (for example following a meal), glucose molecules move into the β

cell via facilitated diffusion through glucose transporter type 2 (GLUT2). Importantly, GLUT2 activity is not dependent upon insulin (3). Next, the high K_m enzyme glucokinase phosphorylates glucose, forming glucose-6-phosphate (Fig. 2). Glucokinase is the rate-limiting enzyme in glucose metabolism, thereby functioning as a glucose sensor and regulator of insulin secretion (4). Glycolysis leads to an increase in intracellular ATP, and subsequent closing of ATP sensitive potassium channels in the β cell plasma membrane (5). This results in membrane depolarization, and opening of voltage gated Ca^{2+} channels. The subsequent increase in cytosolic Ca^{2+} concentration causes insulin-containing vesicles to fuse to the plasma membrane (Fig. 2). In this manner, the β cell directs exocytosis of insulin in appropriate amounts to dispose of blood glucose and suppress hepatic glucose output.

Although the main stimulus for insulin release is an elevation in blood glucose, several other factors can trigger its release, including amino acids, fatty acids, hormones, and neuronal stimuli (3). In addition to its role in GSIS, glucose may also affect insulin production by enhancing stabilization and translational efficiency of *preproinsulin* mRNA (6,7).

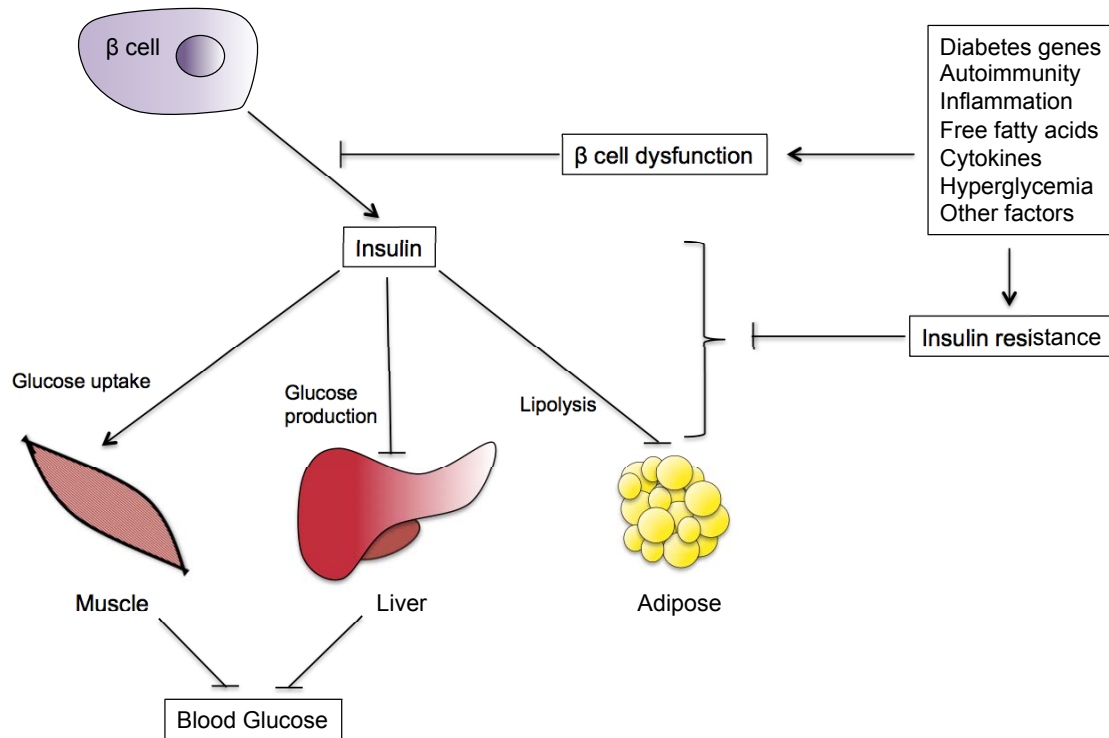


Figure 1: Pathophysiology of diabetes mellitus. Insulin secreted from pancreatic β cells acts to regulate blood glucose concentration. Insulin allows glucose uptake in adipose tissue and skeletal muscle, while inhibiting hepatic glucose output. In the setting of diabetes, a number of factors including genetic predisposition, cytokines, free fatty acids, and hyperglycemia initiate insulin resistance and/or repress β cell function, leading to impairments in blood glucose homeostasis.

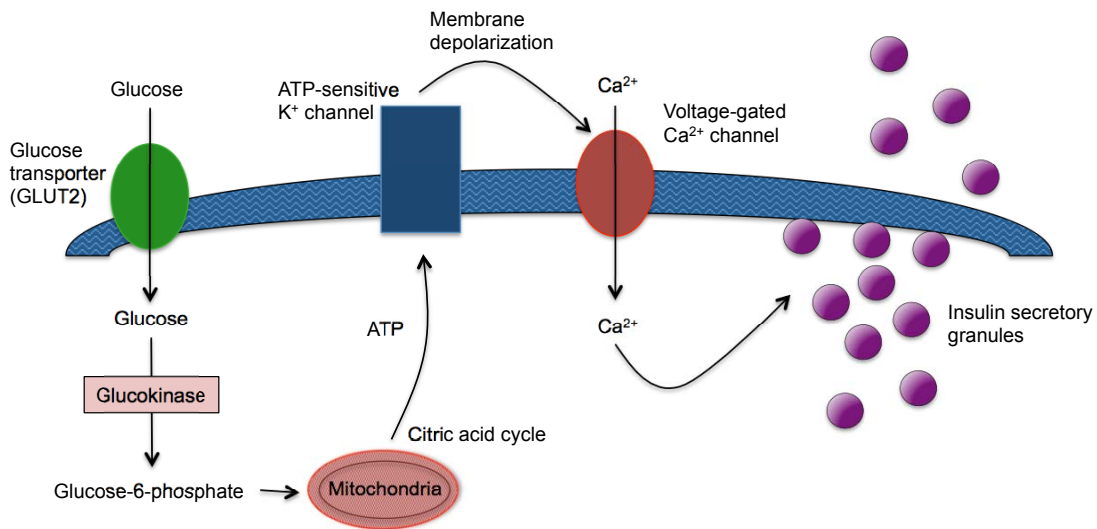


Figure 2: Glucose-stimulated insulin secretion. A schematic representation of GSIS in the pancreatic β cell. Glucose enters the β cell via facilitated diffusion through GLUT2 transporters. Glucokinase catalyzes the rate-limiting step in GSIS, phosphorylation of glucose to glucose-6-phosphate. This leads to further metabolism through glycolysis and the citric acid cycle to generate ATP. Increased intracellular ATP concentration closes ATP-sensitive K^+ channels, leading to membrane depolarization and opening of voltage-gated Ca^{2+} channels. Subsequent influx of Ca^{2+} ions leads to secretion of insulin containing granules via exocytosis, and normalization of blood glucose.

B. Transcriptional Regulation in β Cell Development

In an effort to promote β cell insulin secretion and/or derive new β cells, recent research has focused on understanding the mechanisms directing embryonic β cell development and maintenance of the mature β cell phenotype. These studies have identified a number of β cell transcription factors critical to these processes. These transcription factors are DNA binding proteins that engage consensus sequences in the promoter region of certain genes, thereby regulating transcription of those genes.

The generation of mature β cells from multi-potent progenitors requires spatial and temporal regulation of transcription factor expression. Development of the expression involves at least three essential steps: 1) commitment of endodermal progenitor cells to pancreatic cell fate, 2) differentiation of pancreatic endoderm to endocrine precursors, and 3) commitment of endocrine precursors to β cell fate. Each of these developmental steps is regulated by transcription factor expression (8). A recent study shows these developmental steps can be recapitulated through adenovirus-mediated overexpression of just three factors (Pdx1, Neurog3, MafA) in non- β cell types in the pancreas, with the resultant “reprogrammed” cells phenotypically indistinguishable from mature β cells (9).

Pdx1 is perhaps the most critical protein in pancreas and its development. *Pdx1* is expressed in the early endoderm that is destined to form the pancreas, distal stomach, and duodenum. In the adult, *Pdx1* expression is restricted largely to β cells, although low-level expression is observed in δ cells of the islet, and acinar and duct cells of the pancreas (10). Absence or inactivation

of Pdx1, as observed in *Pdx1*^{-/-} mice and in humans with *PDX1*^{-/-} mutations, results in near-complete pancreatic agenesis (11,12). Moreover, *Pdx1*^{+/-} mice (MODY4, see Chapter 1.2A) exhibit glucose intolerance as a result of impaired insulin release (13). These data indicate a central importance of Pdx1 in pancreas and β cell development.

Neurog3 is a basic helix-loop-helix transcription factor that directs pancreatic progenitor cells towards the endocrine fate (14). *Neurog3*^{-/-} mice have normal exocrine and ductal tissue, but are completely devoid of endocrine tissue (15). Conversely, ectopic *Neurog3* expression throughout the entire forming pancreatic bud (via *Pdx1* promoter-driven transgene) results in near complete conversion of the pancreas into endocrine tissue, consisting mostly of glucagon-producing α cells (16,17). Conversion of endocrine progenitors toward the β Ccell fate requires additional transcription factors. The Maf leucine-zipper containing transcription factors *MafA* and *MafB* direct the concluding steps of β cell development. *MafA*^{-/-} and *MafB*^{-/-} mice develop morphologically normal islets. However, genetic deficiency of either *MafA* or *MafB* results in a reduction in the number of β cells present (18–20). Other transcription factors including *Pax4*, *Nkx6.1*, and *Nkx2.2* are also involved in directing final β cell differentiation (21–23). Together, these transcription factors enable development of functional islet β cells.

C. Transcriptional Regulation in β Cell Function

In addition to its role in pancreas and β cell development, Pdx1 is essential for maintenance of β cell identity and function. In the mature β cell, Pdx1 has been shown to regulate genes involved in *preproinsulin* transcription and secretion, including *preproinsulin* itself, *Glut2*, *Gck* (encoding glucokinase), *MafA*, *Nkx6.1*, and its own gene (*Pdx1*) (24–28). Deletion of *Pdx1* in the β cell results in altered islet morphology, decreased insulin, islet amyloid polypeptide (IAPP), and GLUT2 production, impaired GSIS, and glucose intolerance (24,29). Chromatin immunoprecipitation and promoter microarray studies have revealed a number of additional genes putatively regulated by Pdx1, including those concerned with cell cycle, cell survival, exocytosis, and energy sensing (30).

Recently, Pdx1 has been implicated in maintenance of β cell function in the setting of diabetes. Insulin resistance induced β cell compensatory hyperplasia (discussed in Chapter 1.2D) is attenuated in *Pdx1*^{+/-} mouse models (31). Additionally, Pdx1 heterozygosity predisposes mice to β cell ER stress and apoptosis (32). Finally, loss of Pdx1 in the adult mouse using a tet-off inducible gene repression system results in progressive β cell dysfunction, reduced insulin and GLUT2 production, and hyperglycemia (33).

Studies indicate Pdx1 is essential for β cell health, inviting speculation about whether increasing *Pdx1* expression could lead to preservation or enhancement of functional β cell mass. Indeed, overexpression of Pdx1 in animal models of β cell dysfunction enhances β cell insulin content and GSIS, and promotes β cell mass and glucose tolerance (34,35). Therefore, loss of

Pdx1 expression may also contribute to β cell dysfunction and glucose intolerance in human populations, and strategies to enhance Pdx1 expression may prove valuable as therapies for diabetes.

1.2 ISLET β CELL DYSFUNCTION IN DIABETES MELLITUS

Despite its central importance in regulating energy metabolism, the β cell is subject to a number of stressors that can lead to its dysfunction or death in the setting of diabetes. This is compounded by the fact that β cells have limited numbers and a low inherent replication rate (36). The extent to which an individual's β cells are able to maintain their function may distinguish those who are at risk of developing diabetes from those who are not (37). Indeed, β cell dysfunction and/or failure underlie all forms of diabetes.

Diabetes mellitus comprises a number of disorders resulting from absolute or relative insulin insufficiency, resulting in hyperglycemia (Fig. 1). There are five main subtypes of diabetes: maturity onset diabetes of the young (MODY), type 1 diabetes mellitus, latent autoimmune diabetes of adulthood, type 2 diabetes mellitus, and gestational diabetes mellitus. Although the origins and progression of these diseases vary, β cell dysfunction or death is evident in each. As such, research and therapies aimed at promoting β cell function and survival are critical to promoting health in an increasingly diabetic population.

A. Maturity Onset Diabetes of the Young

Monogenic forms of diabetes are rare diseases characterized by mild hyperglycemia, autosomal dominant inheritance, and onset prior to 30 years of age. MODY results from mutations in genes critical to β cell development and function. Currently, there are six recognized MODY genes, including *HNF4A* (MODY1), *GCK* (MODY2), *HNF1A* (MODY3), *PDX1* (MODY4), *HNF1B* (MODY5), and *NEUROD1* (MODY6) (38,39). Most common among these forms of diabetes are MODY2 and MODY3 (39). In addition to these, several other genes are associated with MODY-like phenotypes, including *KLF11* (a regulator of Pdx1 transcription), *PAX4*, and *preproinsulin* (40).

Another form of monogenic diabetes associated with MODY is permanent neonatal diabetes mellitus. Individuals affected by this disease display a more pronounced phenotype, experiencing severe hyperglycemia within the first weeks of life. Permanent neonatal diabetes mellitus results from autosomal dominant/heterozygous mutations in *KCNJ11* and *ABCC8* genes, or from autosomal recessive/homozygous mutations in glucokinase, *PDX1*, and *PTF1A* (40).

B. Type 1 Diabetes Mellitus

Type 1 diabetes (T1D), also commonly and incorrectly referred to as juvenile diabetes or insulin-dependent diabetes, comprises ~10% of all diabetes cases. T1D results from autoimmune destruction of pancreatic β cells. There is evidence to support an underlying β cell defect in the setting of T1D, which may

lead to β cell autoantigen presentation and subsequent activation of the autoimmune response (41). Whereas the signals initially promoting β cell autoimmunity remain unclear, subsequent presentation and processing of β cell autoantigens, loss of immune tolerance, and generation of autoreactive T and B cells are hallmarks of T1D. Macrophages, T cells, and dendritic cells infiltrate the islet (insulitis), secreting proinflammatory cytokines including interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF- α), and interferon γ (IFN- γ), and allowing cytotoxic T cell mediated β cell destruction (42–44). Proinflammatory cytokines activate signaling cascades that lead to β cell dysfunction and death (discussed in Chapter 1.3).

C. Latent Autoimmune Diabetes of Adults

Latent autoimmune diabetes of adults, also incorrectly called type 1½ diabetes, is a disease characterized by adult onset of autoimmune mediated β cell dysfunction and death. It is characterized by its slowly progressive nature, residual insulin secretion, and common misdiagnosis as type 2 diabetes (T2D). The pathogenesis of this form of diabetes is similar to that of T1D in that insulitis leads to β cell dysfunction and death, however this process takes place at a substantially reduced rate compared with T1D (45).

D. Type 2 Diabetes Mellitus

Type 2 diabetes (T2D) is the most common form of diabetes, comprising up to 90% of all cases. T2D is characterized by loss of insulin sensitivity in peripheral tissues, and is associated with obesity. Notably, it is believed that only 30% of obese, insulin resistant individuals eventually develop T2D, suggesting that other factors contribute to development of diabetes (46). The majority of insulin resistant individual maintain euglycemia by increasing insulin production through a process known as compensatory β cell hyperplasia, which results in an increase in both β cell mass and function (47). During this process, blood glucose concentrations remain normal, and blood insulin concentrations are elevated. In individuals who eventually develop T2D, this compensatory increase in β cell mass and insulin production fails, leading to insulin insufficiency and frank hyperglycemia. Interestingly, genome-wide association studies identifying candidate gene polymorphisms/mutations that confer risk for T2D uncovered genes related to β cell development and function (37,48). Other factors contributing to β cell dysfunction in models of T2D include saturated free fatty acids (FFAs) and proinflammatory cytokines (discussed in Chapter 1.3).

E. Gestational Diabetes Mellitus

Physiological changes related to pregnancy induce a state of insulin resistance similar to that observed in T2D (49,50). In most cases, the β cell responds with compensatory β cell hyperplasia similar to that seen in T2D, resulting in increased insulin production, and maintenance of euglycemia.

However, in approximately 10% of pregnancies, the β cell is unable to effectively compensate, leading to insulin insufficiency, glucose intolerance, and gestational diabetes (51). Those pregnancies complicated by gestational diabetes pose a risk for recurrence of gestational diabetes in future pregnancies, as well as future progression to T2D (52). Notably, recent studies revealed a decline in β cell function in the first year postpartum in women diagnosed with gestational diabetes, suggestive of a role for β cell dysfunction in this form of diabetes (53).

1.3 MEDIATORS OF β CELL DYSFUNCTION

Several factors contribute to the etiology of β cell dysfunction and death, including glucolipotoxicity (54), cytokines released from adipocytes, activated macrophages, and β cells themselves (55,56), and islet amyloid deposition (57,58), among others (see ref. (59) for a review) (Fig. 3). Recently, many of these stressors have come to be viewed as mediators of islet inflammation. As such, inflammation may represent a unified target for diabetes treatment. In the setting of type 2 diabetes, inflammatory mediators including FFAs and proinflammatory cytokines emanate from adipose tissue itself (60). Insulin resistance, which is associated with obesity and T2D, is considered by many to be a result of chronic inflammation (61). Islet amyloid polypeptide (IAPP) deposition is associated with T2D and β cell dysfunction, and its detrimental effects are mediated through its activation of the inflammasome, which promotes maturation of inflammatory cytokines (see Chapter 1.3B) (62). Islet inflammation is also an accepted component of autoimmune destruction of β cells in T1D (63).

Alone, each of these factors can impair β cell function, leading to inflammation and β cell loss. However, in the setting of diabetes, β cells are likely subject to many such factors at a given time.

A. Lipotoxicity

Lipotoxicity arises as a consequence of obesity and high fat diets, triggering impairments in insulin release and eventual β cell apoptosis (64). Circulating saturated FFAs have long been known to affect β cell signaling and function (65). One such fatty acid, palmitate, causes ER stress, oxidative stress, and c-Jun N-terminal kinase (JNK) activation in vivo, all of these promoting inflammation. Palmitate also enhances inflammation via induction of IL-1 β , TNF- α , IL-6, and IL-8 production, as well as activation of NF- κ B signaling in islets (66,67). Elevated glucose and FFA concentrations lead to oxidative stress in islet β cells, which are particularly vulnerable to due to low antioxidant defense (63,68). Oxidative stress results from an imbalance between the presence of reactive oxygen species (ROS) and the cell's ability to clear the reactive species and repair the resulting damage (69). Oxidative stress mediates inflammatory responses in the β cell through activation of JNK, NF- κ B, and p38 mitogen activated protein kinase (MAPK) (70).

Recent studies suggest that FFAs are required for maintenance of normal glucose responsiveness (65,71), raising the possibility that FFAs contribute to elevated insulin secretion in the initial stages of T2D (72). Alternatively, chronic exposure of β cells to FFAs leads to impairments in GSIS, reductions in insulin

gene expression, and β cell apoptosis (73–75). Thus, although FFA induced compensatory β cell function is a beneficial adaptation in the short term, evidence suggests this adaptation can be harmful in the long run.

B. Islet Amyloid Polypeptide

IAPP is synthesized in the β cell and is co-secreted with insulin; however, its physiological role remains unclear (76,77). Similar to amyloid deposition in Alzheimer's disease, IAPP can oligomerize into fibrils, and form islet amyloid deposits, which contribute to islet inflammation (78). Factors associated with amyloid formation include IAPP amino acid sequence, as well as chronically elevated glucose and FFA levels (79). Mouse islets over-expressing amyloidogenic human IAPP exhibit oxidative stress, and this contributes to amyloid-induced β cell apoptosis (58). This amyloid-induced apoptosis is mediated by JNK activation (80). Further, amyloid deposition triggers the NLRP3 inflammasome, a complex which generates mature IL-1 β (62). Thus, islet amyloid deposition represents another factor promoting inflammation, oxidative stress, and β cell death in T2D.

C. β Cell Dedifferentiation

Recently, studies have proposed a role for β cell dedifferentiation in the pathogenesis of diabetes (81,82). Whereas it is appreciated that diabetes is linked to β cell failure, it is unclear whether this failure is attributable primarily to β dysfunction or reduced β cell mass. Studies of mouse models of diabetes have revealed that loss of β cell mass contributes to β cell failure, but interestingly, the driver of this process may not be β cell death, but instead β cell dedifferentiation into α or some other cell type (81,83). In vitro models also demonstrate that β cells undergo dedifferentiation into an insulin-, Pdx1-, and GLUT2-negative form (82). This evidence demonstrates that β cell dedifferentiation is another mechanism of β cell loss in the setting of diabetes (81). Thus, preservation of β cell identity is required for maintenance of β cell mass and function in the setting of diabetes.

D. Proinflammatory Cytokines

Islet β cells are subject to the effects of proinflammatory cytokines in the setting of both T1D and T2D. In states of obesity as seen in T2D, adipose tissue accumulates and undergoes phenotypic changes, becoming a primary source of circulating proinflammatory cytokines that contribute to systemic inflammation (60,84). In the setting of T1D, islet-infiltrating immune cells and the β cell itself produce cytokines, contributing to the pathophysiology of this form of diabetes (55,56).

Proinflammatory cytokines (e.g. IL-1 β , TNF- α , and IFN- γ , referred to hereafter as cytokines) are known to exert a number of detrimental effects on β cells. Cytokines trigger NF- κ B, JNK, p38 MAPK, and STAT1 dependent signaling, and the intrinsic mitochondrial death pathway (55,66,85,86). Signaling through NF- κ B dependent pathways contributes to transcription of *Nos2* mRNA, iNOS protein expression, and nitric oxide (NO) production (87). The resultant oxidative stress impairs glucose oxidation and ATP production, consistent with mitochondrial dysfunction and production of ROS (88). NO also mediates the cytokine induced unfolded protein response (UPR) in β cells (89), possibly via decreased SERCA2 expression and ER Ca²⁺ dysregulation (90). Further, NO is associated with β cell necrosis and apoptosis (91). Cytokines enhance signaling via MAPK cascades, including through JNK and p38 in the β cell (92). Moreover, activation of signal transducer and activator of transcription 1 (STAT1) via TNF- α and IFN- γ leads to Bim activation and β cell apoptosis (93). The intrinsic mitochondrial death pathway is believed to play a central role in mediating cytokine induced β cell death, with calcineurin mediated Bad dephosphorylation and Bax activity contributing significantly (85). Bim, Bad, and Bax are all pro-apoptotic molecules of the apoptosis regulating Bcl-2 family of proteins. Through these pathways and others, proinflammatory cytokines promote β cell dysfunction and eventual apoptosis.

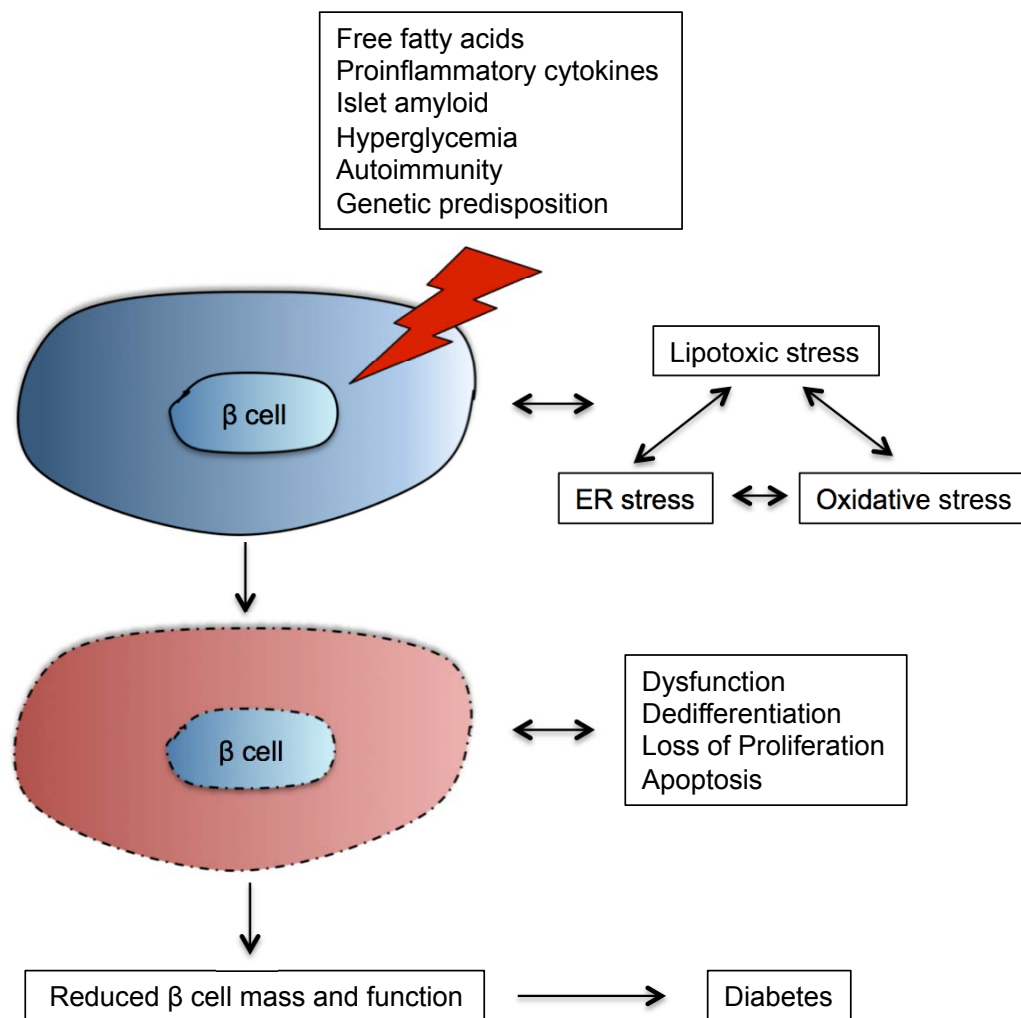


Figure 3: β cell stress leads to reduced β cell mass and function, and diabetes. Factors associated with the diabetic environment including free fatty acids, proinflammatory cytokines, islet amyloid, and genetic predisposition trigger lipotoxic, oxidative, and ER stress in the β cell. These stressors contribute to β cell dysfunction, dedifferentiation, loss of proliferation, and apoptosis. In turn, this leads to reduced β cell mass and function, and diabetes.

1.4 β CELL ENDOPLASMIC RETICULUM STRESS

As a professional secretory cell type, the β cell is particularly dependent upon the integrity of its ER to process, fold, and export insulin. These functions of the ER are tightly regulated and in a state of precarious homeostasis. As such, minor perturbations in the oxidative state of the cell, peripheral tissue insulin demand, and cellular energetics can all impose stress on the ER (discussed in Chapter 1.4B).

A. The Endoplasmic Reticulum

The ER is a eukaryotic organelle consisting of an interconnected membrane network containing luminal and cytosolic surfaces, which are contiguous with the outer membrane of the nuclear envelope. Signal peptides encoded by the first 5-30 amino acids of certain proteins (most notably insulin) associate with a signal recognition particle (SRP), and the ribosome and signal particle complex translocate to the ER membrane (94). Ribosomes associated with the cytosolic surface of the ER membrane participate in the synthesis of secreted and membrane bound proteins, which are translocated into the ER lumen through a translocon (95). Chaperone proteins in the ER lumen, including protein disulfide isomerase (PDI), BiP, calnexin, and calreticulin then aid in the proper folding of these proteins so that they may be exported. Importantly, a number of these chaperones Ca^{2+} dependent, highlighting the importance of Ca^{2+} regulation in the ER lumen (94). The ER lumen is an oxidative environment, allowing formation of disulfide bonds and folding of secretory and membrane

bound proteins (96). In the β cell, insulin synthesis places a high demand on the protein folding capabilities of the ER. In certain scenarios, the demand placed on the ER for protein processing can exceed the ER capacity, leading to the accumulation of unfolded proteins and activation of the unfolded protein response (UPR).

Due to their heavy engagement in synthesis and secretion of insulin, β cells are sensitive to ER stress and subsequent UPR. Prolonged exposure to ER stress may lead to eventual β cell failure and death. Additionally, established β cell stressors such as free fatty acids and proinflammatory cytokines have been demonstrated to cause ER stress (see Chapter 1.3). Models of ER Ca^{2+} dysregulation, such as inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) with thapsigargin, and defective protein processing are also consistent with ER stress (97,98).

B. ER Stress in Diabetes

The association between obesity, insulin resistance, and T2D is well established, and ER stress is one mechanism involved in these processes. In the setting of insulin resistance and T2D, β cells are exposed to high glucose and FFAs, and are compelled to maintain elevated insulin secretion, representing a scenario wherein β cells are subjected to ER stress (99). Importantly, in mouse models of type 2 diabetes, treatment with chemical chaperones reduces ER stress and restores glucose homeostasis (100). It has also been shown that saturated FFAs such as palmitate trigger ER stress in β cells, contributing to

cellular apoptosis in models of T2D (101,102). Palmitate is thought to initially enhance protein synthesis via mTOR activation in the β cell, whereas in the longer run, this activation of protein synthesis contributes to β cell ER stress (103).

Interestingly, recent evidence has implicated ER stress in T1D as well. Type 1 diabetes (T1D) is characterized by loss of immune tolerance to insulin-producing β cells, first leading to impaired insulin production and later to β cell destruction and frank hyperglycemia (104,105). Whereas many studies have focused on the immune system as the primary trigger of T1D, emerging data have pointed to a potentially equally important role of the <http://zotero.org/users/1897719/it>(43,104). Indeed, proinflammatory cytokines secreted by infiltrating immune cells are known to reduce sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2) protein expression, resulting in improper β cell Ca^{2+} homeostasis, and ER stress (90). Recent studies in pre-diabetic non-obese diabetic (NOD) mice (a model of T1D) have suggested that the accumulation of unfolded proteins in the endoplasmic reticulum leads to ER stress prior to onset of frank type 1 diabetes (41,106). It has also been hypothesized that in β cells, ER stress is a major driver of neoantigen exposure, and thereby a trigger for autoimmunity. In support of this hypothesis, NOD mice treated with chemical protein folding chaperones have more robust β cell function and a significantly reduced incidence of diabetes (107). Further, expression of ER stress markers has now been observed in islets of individuals with T1D (108).

1.5 THE UNFOLDED PROTEIN RESPONSE

ER stress leads to activation of the UPR, which initially attempts to remediate stress. Severe or prolonged episodes of ER stress may lead to UPR directed death of β cells, contributing to the development of diabetes (96). During periods of UPR activation, three ER membrane-resident signaling proteins are activated: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PRKR)-like endoplasmic reticulum kinase (PERK), which collectively function to remediate ER stress by promoting protein folding and suppressing new protein production (Fig. 4) (109). The UPR is activated in response to accumulation of unfolded or misfolded proteins in the lumen of the ER. During ER homeostasis, the protein chaperone BiP binds the ER intraluminal portion of PERK, IRE1, and ATF6, maintaining them in a non-active confirmation. During periods of ER stress, unfolded and misfolded proteins compete for more of the available BiP, resulting in its dissociation from PERK, IRE1, and ATF6 (110). This leads to activation of the UPR, which signals initially for adaptation and stress remediation, and later for apoptosis (111). The UPR may also contribute to processes including eukaryotic starvation responses and differentiation programs via nutrient sensing and promotion of ER membrane biosynthesis (112).

A. Activating Transcription Factor 6

Activating transcription factor 6 (ATF6) is a stress inducible ER membrane-resident transcription factor that regulates transcription of UPR target genes, such as the protein chaperone BiP (113). ATF6 differs from most transcription factors in that it is synthesized as a transmembrane protein inserted in the ER membrane. ER stress induces trafficking of ATF6 to the Golgi apparatus, and subsequent proteolysis from the membrane. ATF6 is then free to translocate to the nucleus and facilitate transcription of its target genes (114). ATF6 polymorphisms are associated with impaired glucose tolerance and T2D in human populations, illustrating the relevance of this molecule to human disease (115,116).

B. Inositol-Requiring Enzyme 1

The ER transmembrane protein inositol-requiring enzyme 1 (IRE1) senses ER homeostasis via its interaction with BiP in the ER lumen, and promotes the UPR via its cytoplasmic kinase domain (117). After BiP dissociates from the IRE1 intraluminal domain, IRE1 becomes activated by autophosphorylation, conformational change, and oligomerization (117). IRE1 activation initiates signaling through diverse UPR mechanisms. Notably, IRE1 mediates splicing of a 26 amino acid intron from the transcription factor X-box binding protein 1 (*Xbp1*), leading to expression of Xbp1. In turn, Xbp1 directs transcription of ER biogenesis and protein chaperones genes (118). During the adaptive UPR, IRE1 directs mRNA degradation of multiple substrates through the process of

regulated IRE1-dependent decay (RIDD) in an effort to reduce the translational load placed on the ER (119). If this adaptation is unsuccessful, IRE1 contributes to pro-apoptotic UPR signaling via RIDD of anti-apoptotic pre-miRNAs (120). Furthermore, IRE1's kinase domain phosphorylates JNK, contributing to pro-apoptotic signaling (118). Underscoring the importance of IRE1 in regulating protein synthesis, IRE1 mutation is embryonic lethal at day E9.5-10.5 (121).

C. PRKR-Like Endoplasmic Reticulum Kinase

Protein kinase R (PRKR)-like endoplasmic reticulum kinase (PERK), another ER transmembrane protein, comprises the third arm of the UPR. Similar to IRE1, PERK is activated when BiP dissociates from its ER luminal domain, leading to oligomerization and autophosphorylation (117). PERK has been implicated in regulation of endoplasmic reticulum associated degradation (ERAD), a mechanism that targets misfolded proteins in the ER lumen for ubiquitination and subsequent proteosomal degradation (122). Additionally, PERK plays a critical role in protein trafficking and secretory pathway quality control (123).

A primary function of PERK is to phosphorylate the translation initiation factor eIF2 α . During the UPR, the phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) by PERK causes suppression of general protein synthesis (124). This is an adaptive mechanism to decrease the translational load placed on the ER (125). Whereas general protein synthesis is attenuated under conditions of eIF2 α phosphorylation, translation of certain mRNAs such as

Atf4 and *Chop* is enhanced (126,127). Distinct mechanisms of translational initiation allow this specific regulation of protein synthesis to occur during ER stress (discussed in Chapter 1.6A). Despite its initial adaptive function, excess phospho-eIF2s is poorly tolerated in β cells, exacerbating apoptosis (128). Alternatively, mutation of PERK in mice and in human populations leads to infancy onset of diabetes (129,130). Whereas many studies have elucidated the transcriptional responses of the UPR (downstream of ATF6 and IRE1), fewer studies have investigated the translational responses of the UPR (downstream of PERK) in the β cell.

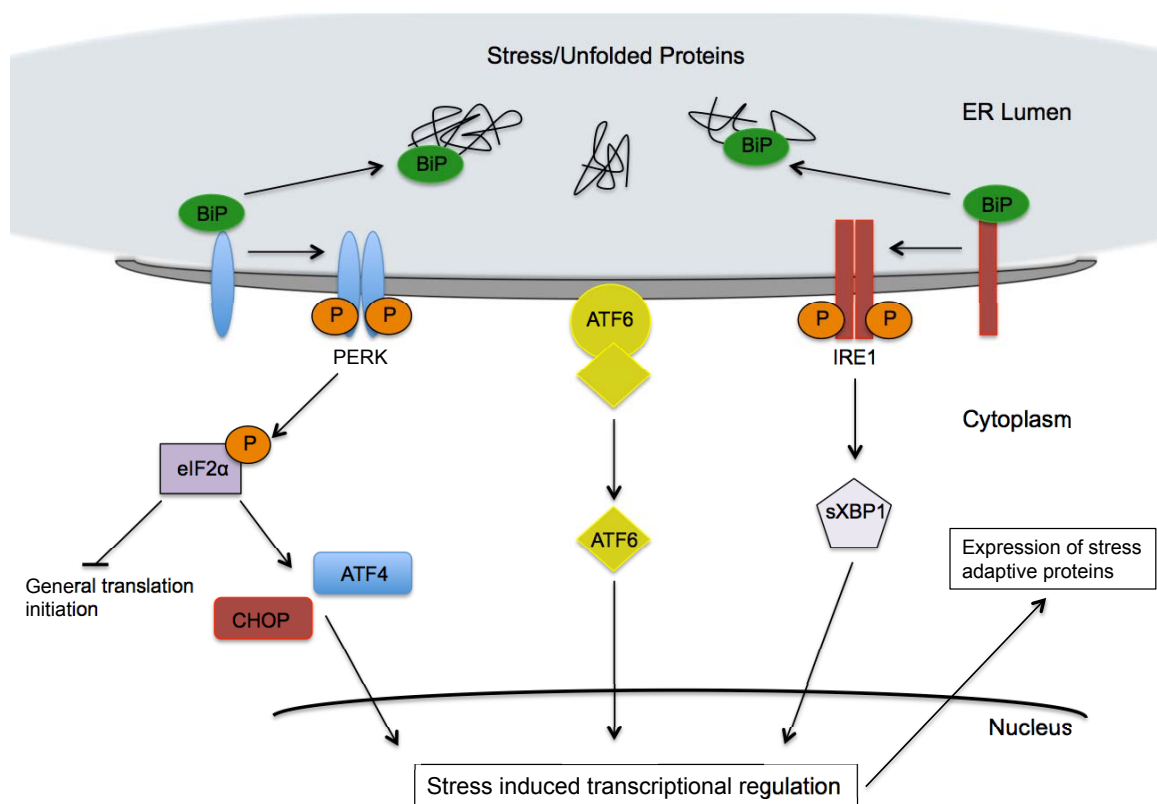


Figure 4: The unfolded protein response. The unfolded protein response is activated in response to accumulation of unfolded proteins in the lumen of the ER. During periods of ER stress, the folding chaperone BiP is recruited away from PERK, ATF6, and IRE1, resulting in activation of these signaling intermediates. ATF4, CHOP, ATF6, and XBP1 proteins are generated, driving transcriptional responses intended to expand ER protein folding capacity and to remediate ER stress. Translational regulation following eIF2 α phosphorylation is also active during the UPR. General protein synthesis is inhibited by phospho-eIF2 α , while translation of a specific subset of transcripts is enhanced or maintained (*Atf4*, *Chop*).

D. The Role of Pdx1 in β Cell ER Homeostasis

Recently, studies have shed light onto the importance of Pdx1 in maintaining by phospho-eIF2 genera(32). Whereas mutation of the Pdx1 gene in both mice and humans results in pancreatic agenesis, Pdx1 heterozygosity leads to MODY4 (see Chapter 1.2A) (13). *Pdx1*^{+/-} mice fed a high fat diet show heightened susceptibility to ER stress owing to failure in the UPR (32). Pdx1 plays a key role in the UPR by directly promoting transcription of *Atf4*, *Wfs1*, and *Ero1b*, among others (32). Thus, in both type 1 and type 2 diabetes, Pdx1 levels may serve as a barometer of β cell ER stress susceptibility. Interestingly, Pdx1 mRNA and protein levels are reduced in NOD mice, which correlated with deficiencies in islet glucose responsiveness in vitro and elevated levels of *Bip* mRNA, *spliced Xbp1* mRNA, and serum proinsulin compared to control mice (41). These findings may be related to an inherent genetic feature of the NOD β cell, and may offer insight into the susceptibility of mice on the NOD background to , cell dysfunction. Therefore, Pdx1 may represent a factor unique to the β cell that is required for maintenance of ER homeostasis.

1.6 MESSENGER RNA TRANSLATION IN THE ISLET β CELL

Studies into β cell translational regulation are critical to understanding β cell dysfunction and death in the setting of diabetes. In addition to translational regulation downstream of ER stress and eIF2 α phosphorylation, many mechanisms involving availability, activity, and assembly of translation factors have been linked to diabetes. Among these are translation initiation factors such as eIF4G and eIF4E, and translation elongation factors such as eIF5A (131–133). Moreover, studies of global mammalian gene expression have revealed a lack of correlation between transcript and protein abundance in cells (134,135). Although mRNA and protein stability may also contribute to this phenomenon, the data suggest that protein expression is regulated principally at the level of translation. However, relatively little research has focused on translational responses of the islet β cell to diabetes-related stressors. The studies conducted here address this gap in knowledge.

Translation is the process by which ribosomes create proteins from an mRNA template. Messenger RNAs produced via gene transcription are translated by ribosomes using aminoacyl tRNAs to produce specific amino acid polypeptides, which then fold to form an active protein (136,137). Eukaryotic translation proceeds through the regulated processes of translation initiation, elongation, and termination (Fig. 5). Each of these processes utilizes specific translation factors that are necessary for accurate and efficient mRNA translation (136,137). Translation is regulated primarily at the level of initiation. However,

recent evidence suggests there is significant regulation at the level of translation elongation as well (133,138,139).

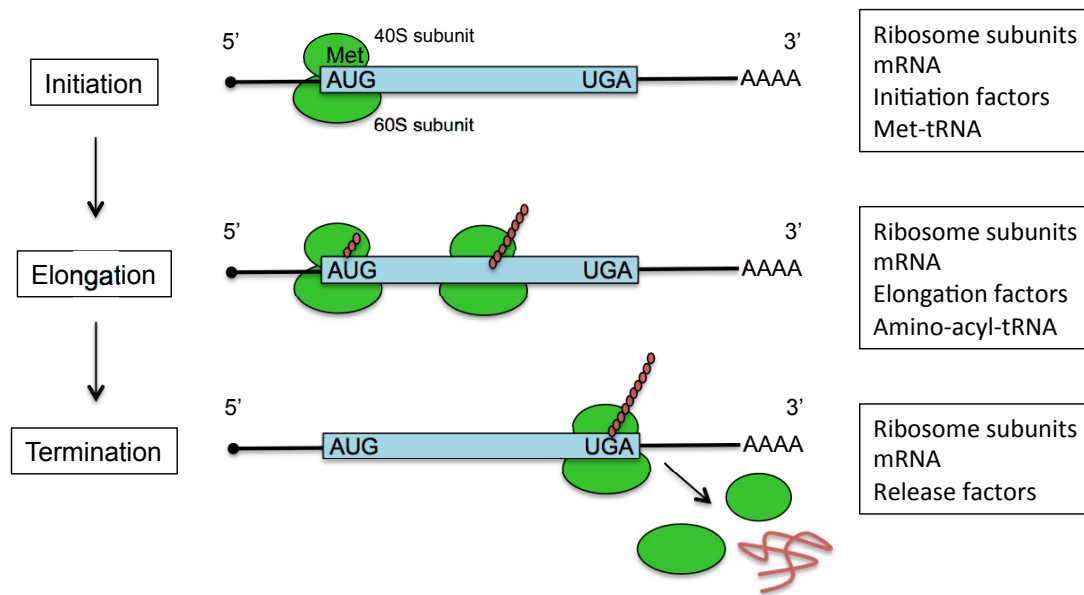


Figure 5: Processes of eukaryotic translation. Eukaryotic translation takes place through three coordinated processes, initiation, elongation, and termination. Translation initiation requires mRNA, ribosome subunits, eIFs, and Met-tRNA; elongation requires ribosome subunits, mRNA, eEFs, and aminoacyl tRNAs; termination requires ribosome subunits, mRNA, and eRFs. Together these processes initiate translation at a start codon, polymerize the growing polypeptide, release the protein for processing and activity, and recycle ribosome subunits and translation factors for use in subsequent cycles of translation.

A. Translation Initiation

Translation initiation is believed to be the most critical process in regulating translation, and it is the most well studied event in translation (137). Most cellular mRNAs are translated via a 5'-7-methylguanylate cap dependent mechanism (Fig. 6). This mechanism allows assembly of eukaryotic translation initiation factors (eIFs) and the ribosomal preinitiation complex at the 5' cap, facilitating scanning by ribosomes to the translation initiation start site (137). The eIF4F cap-binding complex is composed of eIF4E, which binds the mRNA 5' cap, eIF4G, a scaffolding protein linking the mRNA cap to the ribosome preinitiation complex, and eIF4A, an ATP-dependent RNA helicase. The 43S ribosome preinitiation complex is comprised of a 40S ribosomal subunit, an eIF2-GTP-Met-tRNA ternary complex, eIF3, eIF1, and eIF1A (140). Association of this complex with the mRNA 5' cap allows 5' to 3' scanning of the 43S ribosome to reach the initiation codon. Once the 43S complex recognizes the initiation codon and the 48S complex is formed, eIF2 bound GTP is hydrolyzed by eIF5 and eIF5B. This leads to displacement of eIFs and joining of the large, 60S ribosome subunit to form a fully translation competent ribosome (137).

Translation initiation can be regulated via eIF2 α phosphorylation, availability and assembly of initiation factors required for cap-dependent translation, by mRNA binding proteins, and importantly, by varied mechanisms involving the sequence of the mRNA 5' untranslated region (UTR) (137,140–142). One of the most fully understood models of β cell stress-induced translational regulation involves unfolded proteins in the ER lumen activating

PERK, which subsequently phosphorylates eIF2 α (Fig. 4). The guanine nucleotide exchange factor (GEF) eIF2B is maintained in an inactive conformation during periods of high eIF2 α phosphorylation, impairing GDP to GTP exchange on eIF2 α , thus inhibiting global eIF2 α dependent translation initiation (137). This adaptive response decreases the translational load placed on the ER. Essential for eventual stress adaptation, GADD34 is a stress inducible protein phosphatase that directs the dephosphorylation of eIF2 α (143).

While translation of most proteins is down regulated during ER stress and eIF2 α phosphorylation, certain mRNAs necessary to mount an appropriate response to stress are translationally up regulated under these conditions. Key to the mechanisms allowing such translational enhancement are inhibitory upstream open reading frames (uORFs) in the 5' UTRs of these mRNAs (127,142,144). Many mRNAs relevant to β cell stress response are believed to utilize inhibitory upstream open reading frames, including *Atf4*, *Chop*, *Atf5*, and *Gadd34* (144). During ER homeostasis, relatively little phosphorylated eIF2 α is present, and initiation at start codons is very efficient. Under such conditions, inhibitory upstream open reading frames in the mRNA 5' UTR capture scanning preinitiation complexes, initiating protein synthesis of short peptides, and preventing ribosome access to the downstream protein coding ORF. Under conditions of ER stress, when phosphorylated eIF2 α levels increase, initiation at start codons becomes less efficient. Therefore, scanning ribosomes are less likely to initiate translation at inhibitory uORFs, increasing the frequency of translation initiation at the main, protein coding, ORF (145). Through this

mechanism, mRNAs necessary for stress adaptation activate their translation under appropriate conditions. Although such mechanisms are generally accepted for all cell types, these specific mechanisms of translational activation have not been fully examined in the β cell. In the following studies, we assess the function of these mechanisms in β cells using techniques to investigate translational regulation.

Internal ribosome entry sites (IRESs) represent another mechanism allowing maintenance of translation initiation under conditions of stress. IRESs are RNA elements found in the 5' UTR that permit end independent recruitment of ribosomes to maintain or enhance translation initiation under periods of stress (141). Importantly, cellular mRNAs containing IRESs may also be translated via a cap-dependent ribosome scanning mechanism, raising questions about when one or the other mechanism predominates (137). Although IRESs are associated with mRNA secondary structure, there is little conservation of structure among known IRESs, and precise mechanisms of action are not well defined. However, eIF4G and eIF4A are believed to be necessary for activity of many cellular IRESs (137). Elevated eIF4G expression in certain breast cancers strongly drives IRES mediated translation of p120 catenin and of vascular endothelial growth factor (VEGF) mRNA (146). IRES trans-acting factors (ITAFs), RNA binding proteins postulated to stabilize optimal IRES conformation, also participate in regulation of IRES mediated translation initiation (147). Although transcripts relevant to β cell stress response, including *Bip* and *Xiap*, are known to utilize IRES elements, these types of translational regulation have

not been investigated in the β cell (148,149). The following studies represent some of the first to investigate the relevance of IRES mediated translation to β cell physiology.

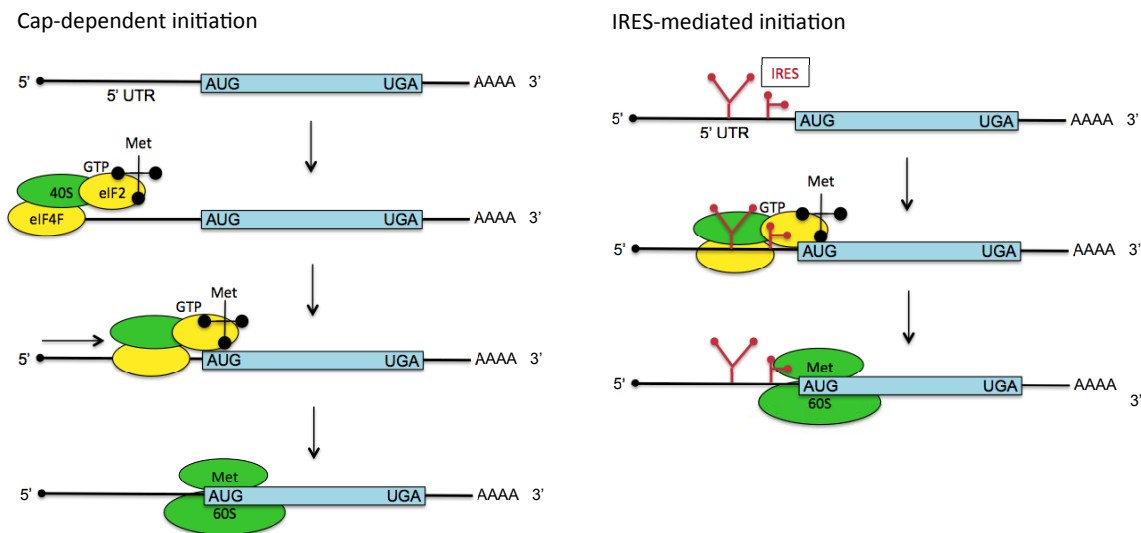


Figure 6: Translation initiation. In cap-dependent translation initiation, the small ribosomal subunit (40S) is recruited to the 5' cap structure through its interaction with the eIF4F cap binding complex. The ribosome then “scans” the 5' UTR until it encounters a start codon (AUG). Codon-anticodon interaction and initiation factors contribute to start codon selection. Several factors are left out of the schematic for clarity. Internal ribosome entry site (IRES)-mediated translation initiation allows assembly of the ribosome complex independently of the 5' cap structure. After recognition of the start codon, binding of the large ribosomal subunit (60S) forms a functional ribosome. This process involves hydrolysis of eIF2 bound GTP, and release of initiation factors.

B. Translation Elongation

Following translation initiation, several mechanisms are involved in elongation of the forming polypeptide. Central to this process are eukaryotic translation elongation factors (eEFs), including eEF1A and eEF2 (150). When an 80S ribosome is positioned on an mRNA with the anticodon of Met-tRNA base paired with the start codon in the ribosome P-site, initiation is complete and elongation may proceed. At this stage, the second codon of the ORF waits for its cognate aminoacyl tRNA in the ribosome A-site. Elongation factor eEF1A then binds aminoacyl-tRNA, directing the tRNA to the ribosome A-site in a GTP dependent manner. Codon recognition by this tRNA then prompts eEF1A mediated GTP hydrolysis, releasing the elongation factor and completing tRNA association with the A-site (150). Following accommodation of the tRNA in the A-site, peptide bonds are rapidly formed with the peptidyl-tRNA of the P-site. Critical to this mechanism is the ribosome peptidyl transferase center (PTC), which consists predominantly of rRNA elements of the 60S subunit, and functions to optimally position substrates for catalysis (151). After peptide bond formation, translocation of tRNAs to the E-site and P-site is catalyzed by elongation factor eEF2. Hydrolysis of GTP by eEF2 at the ribosome leads to conformational changes to eEF2 and the ribosome, which are thought to allow uni-directional translocation of tRNAs through the ribosome (150). Now, the peptidyl-tRNA occupies the P-site, and the A-site is once again vacant, awaiting the next aminoacyl-tRNA-eEF1A complex.

The process of translation elongation is regulated by mechanisms involving a variety of elongation factors. For example, following hydrolysis of eEF1A associated GTP, the GDP bound eEF1A complex must undergo nucleotide exchange to reacquire GTP and activity. Thus, eEF1B, which catalyzes guanine nucleotide exchange of eEF1A, plays a key role in regulating eukaryotic translation elongation (150). Additionally, the translational activity of eEF2 is regulated by post-translational modification. Phosphorylation of eEF2 by eEF2 kinase (eEF2K), results in impaired eEF2-ribosome interaction, and blockade of translational elongation (152,153).

Interestingly, eEF2 is subject to a unique post-translational modification impacting translation. A conserved His residue of eEF2 undergoes a unique post-translational modification to form the amino acid diphthamide (154). Mutation of this eEF2 residue results in impaired cell growth, and mice lacking enzymes required for diphthamide formation are subject to embryo lethality or severe developmental defects. These data indicate a positive role for diphthamide in translation (155,156). The ability of external stimuli to regulate function of translation elongation factors via these processes is an incompletely understood area of translation regulation.

C. Elongation Factors eIF5A and DHS

Eukaryotic translation initiation factor 5A (eIF5A), is a small (17 kDa) protein that is highly conserved throughout evolution and is expressed in a broad range of cell types in mammals (157). Originally termed a translation initiation

factor due to its ability to stimulate methionyl-puromycin synthesis, eIF5A is now thought to function primarily as a translational elongation factor (158). Depletion of eIF5A in yeast and mammalian cells results in the accumulation of polyribosomes and in prolonged ribosome transit times (158,159). Importantly, loss of eIF5A in yeast results in a ~30% decrease in protein synthesis rates (160). However, in unstressed mammalian cells, it has been reported that depletion of eIF5A results in the impaired translational elongation of only about 5% of mRNAs (159). Both of these findings argue against a role for eIF5A as a general translation factor, and instead point to a more restricted role in the translation of a subset of mRNAs (161,162). Studies into the mechanisms of eIF5A have proposed a model whereby active eIF5A serves to catalyze peptide bond formation between unfavorable amino acid substrates such as polyproline motifs, reducing ribosome stalling on mRNA (138,139). Intriguingly, other data suggests that ribosome stalling on certain eukaryotic transcripts contributes to IRES mediated translation initiation (163). Thus, there may be a link between IRES activity and hypusinated eIF5A in translational regulation.

Similar to the diphthamide modification of eEF2, eIF5A also undergoes an unusual posttranslational modification, forming hypusine. Hypusine is required for virtually all of the functions of eIF5A studied to date, including RNA binding, mRNA shuttling, and translational elongation (164). Hypusine synthesis is catalyzed by the sequential actions of deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH), which together transfer the polyamine moiety of spermidine to the neine moiety of spermidineentia (Fig. 7) (165).

DHS catalyzes the rate-limiting step in the hypusine biosynthetic pathway, and as such, represents a target for inhibiting the overall rate of hypusine formation. Inhibition of hypusination may attenuate translation of specific mRNAs involved in β cell dysfunction. Several inhibitors of DHS have been described, all classified as polyamines with structural homology to spermidine. Perhaps the best studied and most potent inhibitor is *N*¹-guanylyl-1,7-diaminoheptane (GC7), which exhibits a *K_i* of 10 nM *in vitro* (about 450-fold lower than the *K_m* for spermidine) (166). X-ray crystallographic analysis of DHS at near-optimal pH and ionic conditions shows that GC7 is specifically bound within a deep acidic active site tunnel (167). Inhibition of DHS using GC7 and similar polyamines was shown to have a repressive effect on proliferation in both yeast and mammalian cell cultures as soon as 24h, with a particularly striking inhibition at the G1/S transition of the cell cycle (168–170). Given its role as a translational regulator of stress responsive mRNAs, it is interesting to speculate about what types of signals may activate or repress hypusine mediated eIF5A activity, what the mRNA targets of eIF5A are, and how pharmacological and genetic models of DHS insufficiency affect β cell function and survival. In the studies outlined herein, I investigated mechanisms of hypusine-eIF5A mediated translational elongation in the islet β cell.

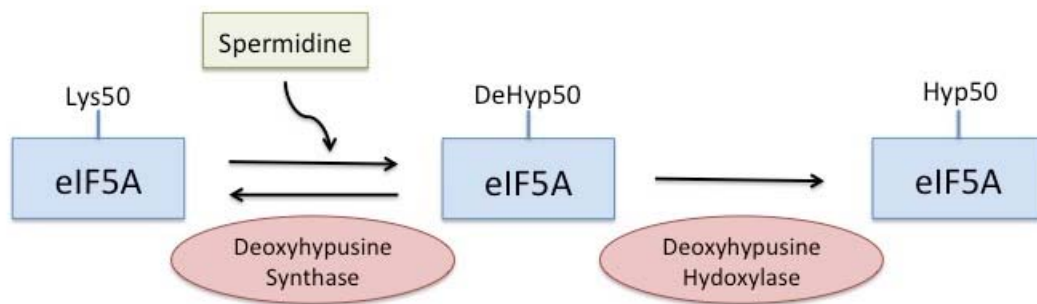


Figure 7: Hypusination of eIF5A. Deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) act sequentially to form a hypusine residue at lysine 50 of eIF5A. The small molecule GC7, a spermidine analog, inhibits DHS function, suppressing eIF5A hypusination.

D. Translation Termination

Termination is perhaps the least well studied of the three stages of translation. This process occurs when the ribosome reaches a stop codon at the end of a protein coding sequence, and utilizes eukaryotic translation release factors (eRFs) (150). Eukaryotic termination is directed cooperatively by eRF1, eRF3, and ABCE1/Rli1. After recognition of the stop codon, the eRF1-eRF3-GTP complex binds the ribosome A-site, leading to eRF3 mediated GTP hydrolysis and subsequent eRF3 release (171,172). ABCE1/Rli1 then facilitates accommodation of eRF1 into optimal configuration, leading to peptide release and subunit dissociation (150). Release factor eRF1 is essential for ABCE1 activity, which promotes dissociation and recycling of post-termination complexes into free ribosomal subunits (173). Although relatively little is known about regulation of translation termination, processes of subunit and translation factor recycling play significant roles in maintaining efficient translation.

1.7 SUMMARY

Islet β cell research has now provided convincing evidence that loss of β cell function is the key determinant in development of both type 1 and type 2 diabetes (42,59). As such, research focusing on maintenance of β cell health and function is well positioned to provide insights for development of new therapeutic strategies. While β cell stress responses are generally well understood, little is currently known regarding β cell translational regulation, which is a likely consequence of inflammation, ER stress, and the UPR. My

research has focused on defining the translational regulatory mechanisms that are operable in the islet β cell, and shedding new light on how these may be exploited for the treatment of diabetes.

Studies into stress induced translational changes will likely provide new insight for improved β cell function and survival. Mechanisms anticipated to be relevant to β cell physiology include regulation of global translation initiation via phosphorylated eIF2 α and 5'-7-methylguanylate cap components. Also, transcript specific regulation of translational initiation (*Atf4*, *Chop*) and elongation are likely contributors to β cell stress response (see Chapter 1.7). Careful study of these proposed mechanisms of translational regulation will establish their relevance to β cell physiology and add to the current understanding of β cell stress response.

In addition to validating targets of translational regulation in the β cell, the study of translational regulation of β cell specific transcripts will provide critical insight into how β cells maintain homeostasis under conditions of stress. Recent data indicate Pdx1 is required for maintenance of ER homeostasis under conditions of β cell stress. Indeed, Pdx1 is a direct transcriptional regulator of *Atf4*, *Ero1b*, and *Wfs1*, and is linked to β cell Ca^{2+} homeostasis (32). Since Pdx1 is a key contributor to the UPR, I suggest that maintenance of Pdx1 mRNA translation in the setting of ER stress is important for the adaptive stress response in β cells.

A primary goal of these studies was to determine the translational changes associated with β cell stress, including proinflammatory cytokines. I

hypothesize that translational regulation is a key component of the pancreatic β cell stress response. Further, I hypothesize that Pdx1 mRNA maintains translation during ER stress, and that DHS and eIF5A are required for translational elongation of β cell transcripts involved in stress response. To test these hypotheses, and to better understand β cell translational responses in general, I established cell culture systems in vitro that recapitulate the translational changes seen during ER stress. I then investigated transcriptional, translational, and post-translational mechanisms related to translational regulation in the islet β cell.

CHAPTER 2

Experimental Procedures

2.1 MATERIALS

A. Animals, Islets, and Cell Lines

Mice (C57BL/6) were bred and maintained at the Indiana University Laboratory Animal Resource Center under pathogen free conditions according to protocols approved by the Institutional Animal Care and Use Committee. The mouse insulinoma cell line MIN6 was maintained in high glucose DMEM with 15% FBS, 1% Pen/Strep, and supplemented with HEPES and sodium pyruvate. The rat insulinoma cell line INS1 (832/13) was maintained in RPMI with 10% FBS, 1%Pen/Strep, and supplemented with HEPES. MEFs were maintained in DMEM supplemented with 10% FBS and 1% Pen/Strep following isolation. Briefly, one male and two virgin female mice were housed in each cage, and females were checked for copulatory plugs each morning until observation. Then, plugged females were removed to a separate cage. Embryos were harvested at day E8.5 or E13.5 from euthanized pregnant females, washed with PBS, minced in 1X trypsin, and cells were allowed to grow onto tissue culture plastic at 37°C and 5% CO₂. Mouse islets were isolated from collagenase-perfused pancreata as previously described (174). Islets were picked by hand and counted, and then allowed to recover overnight in 11mM glucose RPMI before experimentation. Human islets were obtained through the Integrated Islet

Distribution Program. Upon arrival, human islets were placed in RPMI and allowed to recover overnight before experimentation.

B. Antibodies

Primary antibodies previously described include anti-slet Distribution Program. Upon arrival, anti-eIF5A, anti-eIF2A, anti-phospho-eIF2s, anti-p38, and anti-phospho-p38 (88,103,162,175). Other primary antibodies used in these studies include anti-iNOS (06-573, Millipore or SC-8095, Santa Cruz Biotechnology), anti-Pdx1 (07-696, Millipore), and anti-CPE (11044, Abcam). Fluorophore labeled secondary antibodies IRDye 800 and IRDye 700 were purchased from Li-Cor Biosciences (Lincoln, NE).

C. Vectors

The C49 dicistronic vector construct including the hSP-A 5' UTR insert (C49 +, a gift from Patricia Silveyra and Joanna Floros) (176) was digested with EcoRI (New England Biolabs), separated on a 2% agarose gel, and the backbone and insert were gel purified (Qiagen). PCR amplification of the hsPdx, mmPdx1, mmPdx1 truncation mutant, and CPE 5' UTRs was carried out using the following primers. For hsPdxUTR, 5'- AAAGCGAGCAGGGTGGCG-3' (forward) and 5'- GGCTGCGGCCCGGGATT-3' (reverse) were used. For mmPdxUTR, 5'- AAAATTGAAACAAGTGCAGGTG-3' (forward) and 5'- GGTGGCAGCCGGCACTTG-3' (reverse) were used. For mmPdx1UTR truncation mutant, 5'- GTCAAAGCGATCTGGGGTG-3' (forward) and 5'-

GGTGGCAGCCGGCACTTG-3' (reverse) were used. For CPE-UTR, 5'-GTGAGGCGAGAGGAGGCTGGTGCTG-3' (forward) and 5'-CGCGTCCCCGCGAGCTGCACTGCC-3' (reverse) were used. These PCR products were TA cloned into pCR2.1 (Invitrogen) and transformants were screened for inserts. The pCR2.1 vector carrying 5' UTR insert was then digested with EcoRI, separated on a 2% agarose gel, and the inserts were gel purified (Qiagen). These 5' UTR inserts were then ligated to EcoRI cut C49 backbone, and colonies were screened for inserts. Colonies lacking an insert were sequenced and used as a negative control (C49 -). Vectors containing the 5' UTR insert were sequenced to determine correct directionality of the ligated insert (Operon). Finally, point mutations were introduced to the C49 mmPdxUTR construct to eliminate uORFs using a Site Directed Mutagenesis kit (Agilent) to mutate upstream ATG codons to AGG codons.

2.2 METHODS

A. Cell Cycle Analysis

Approximately 10^6 cells were harvested by trypsinization, washed twice in PBS, and resuspended in ice-cold 70% ethanol. Cells were then washed twice in PBS, resuspended in Guava cell cycle reagent (Guava Technologies), and incubated at room temperature for 30 minutes. Cell cycle phase was then determined via FACS analysis using a Beckman Coulter FC500 flow cytometer and FloJo data analysis software.

B. Cytokine and Inhibitor Treatment

Islet or cell cultures were treated at 37°C with a cytokine cocktail containing 5ng/ml IL-1 β , 10ng/ml TNF- α , and 100ng/ml IFN- γ (Prospec) for the indicated times. Cell cultures and islets were treated with 1 μ M thapsigargin (a SERCA inhibitor and inhibitor of translation initiation) at 37°C for the indicated times. Inhibitors to MAPKs p38 (PD169316) and JNK (SP600125) were used at a final concentration of 10 μ M, the iNOS inhibitor L-NMMA was used at a final concentration of 1mM, and the DHS inhibitor GC7 (see Chapter 1.6C) was used at a final concentration of 100 μ M. Cycloheximide (CHX), an inhibitor of translational elongation, was used at a concentration of 50 μ g/ml.

C. Dual Luciferase Assay

Following transfection of luciferase expressing constructs, luciferase activity was determined using a commercially available dual luciferase activity kit (Promega) and luminometer (Turner BioSystems). Approximately 10⁶ luciferase transfected MIN6 β cells were washed with PBS, then lysed in 500 μ l passive lysis buffer for 15min at RT, spun at 13,000 x g for 1min, and the cleared lysate was moved to a new tube. Luciferase assay reagent II (LARII) and Stop & Glo reagent (Promega) were used to prime the luminometer. Using a luminometer program (Turner BioSystems), 100ul of LARII was added to 20 μ l of cell extract, and firefly luciferase activity was evaluated. Next, 100ul of Stop & Glo reagent was then added, and renilla luciferase activity was determined.

D. Glucose Tolerance Test

Mice were fasted overnight and injected intraperitoneally with 2g glucose/kg body weight. Blood was sampled from the tail vein at 0, 10, 20, 30, 60, 90, and 120 minutes post injection, and blood glucose was measured from whole blood using the AlphaTrak blood glucose monitoring system (Abbott Laboratories).

E. ^3H -Spermidine Incorporation Assay

To assess the activity of DHS in vitro, we measured ^3H -spermidine incorporation into hypusine-eIF5A. Approximately 100 islets or 10^6 MIN6 or INS1 β cells were incubated with 1.5 μCi [^3H] spermidine (PerkinElmer) per ml culture for 4h at 37°C. Whole cell extracts were then isolated and subjected to 12% SDS-PAGE. Gels were fixed for 30min in a solution containing 10% acetic acid and 30% methanol, transferred to En 3 HANCE autoradiography enhancer solution (Perkin Elmer) for 1h, and to 10% polyethylene glycol for 30min, dried for 1h in a gel dryer and vacuum pump (Biorad), and visualized by fluorography. Band intensity was quantified using ImageJ Software (NIH).

F. ^3H -Thymidine Incorporation Assay

Proliferation was assessed via incorporation of ^3H -thymidine into genomic DNA of MEFs. Approximately 10^6 cells were incubated with 1 μCi [^3H] methyl-thymidine (Perkin Elmer) for 4h, washed with cold PBS, and DNA was precipitated with cold 10% trichloroacetic acid for 15min, and solubilized with

0.3N NaOH for 30min. Amount of [^3H] thymidine incorporation was measured using liquid scintillation counting, and was normalized to protein content.

G. ^{35}S -Cystein/Methionine Incorporation Assay

For ^{35}S -Cys/Met uptake assays, approximately 10^6 MIN6 cells were cultured in 6 well plates in the presence or absence of cytokines for 24 or 72 h, then 100 μCi of a mixture of ^{35}S -Met and ^{35}S -Cys (Perkin Elmer) was added for one hour. The medium was removed, and cells were washed 3 times with PBS containing 1 mM Met and 1 mM Cys (Sigma), then lysed in SDS loading buffer. A fraction of the lysate was counted on a scintillation counter to correct for ^{35}S amino acid uptake, and corrected lysate volumes were subjected to 4-20% SDS-PAGE. The gel was dried as discussed previously, and visualized by fluorography.

H. Immunoblot Analysis

Whole cell extracts from MIN6 β cells, INS1 β cells, and islets were collected in lysis buffer containing 10% glycerol, 0.1% NP-40, 0.05% deoxycholate, 50mM Tris-HCl, 10mM NaF, 1mM EDTA, and 1mM DTT and supplemented with protease and phosphatase inhibitors (Roche). Immunoblot analyses of cell extracts were performed after separation of 15 μg of protein extract by 4-20% gradient SDS-PAGE (Invitrogen). Protein was transferred to a fluorescence compatible PVDF membrane (Millipore) using a wet transfer apparatus (BioRad). Membranes were blocked with Odyssey blocking buffer

(LiCor Biosciences), and incubated overnight at 4°C with primary antibodies. Immunoblots were then washed with 0.05% Tween 20 in PBS, incubated with fluorophore labeled secondary antibodies for 1h at RT, and washed with PBS. Immunoblots were visualized using a Li-Cor Odyssey fluorescent imaging system (Li-Cor Biosciences) and blot intensity was quantified using ImageJ software.

I. Polyribosomal Profiling

PRP experiments in MIN6 β cells, INS1 β cells, and islets were conducted as described previously (41). Cells were grown to 70% confluency in 100mm tissue culture dishes, and washed twice with cold PBS containing 50 μ g/ml cycloheximide (CHX, Sigma-Aldrich) and harvested in 500 μ l of lysis buffer containing 20mM Tris-HCl (pH 7.5), 10mM MgCl₂, 100mM NaCl, 1% Triton X-100, 50U/mL recombinant ribonuclease inhibitor RNasin (Promega), and 50 μ g/ml CHX. The cell lysates were passed through a 23-gauge needle and incubated on ice for 10 min, followed by centrifugation at 13,000 x g for 10 min at 4°C. A portion of the lysate supernatant was preserved as the input sample to determine total mRNA levels. Supernatant (400 μ l) was then added onto a 10-40% sucrose gradient solution containing 20mM Tris-HCl (pH 7.5), 5mM MgCl₂, 100mM NaCl, and 50 μ g/ml CHX. The sucrose gradients were subjected to centrifugation at 4°C in a Beckman SW-41Ti rotor at 40,000rpm for 2h. A piston gradient fractionator (BioComp) was used to fractionate the gradients, and absorbance of RNA at 254 nm was recorded using an in-line UV monitor (BioRad). The eluate

was collected using a fraction collector, and total RNA from the fractions was purified (Qiagen), reverse transcribed, and subjected to qRT-PCR.

J. Transfection

Transient transfections were performed using 70% confluent MIN6 β cells in a 6-well tissue culture dish. For translation studies, 2 μ g of reporter plasmid (C49 constructs) was combined with 6 μ l of Metafectene reagent (Biontex) and added to cells in Pen/Strep free media for 6h. Transfection media was then removed, and growth media was returned to the cultures. Cells were harvested 24h after transfection, and prepared for either RNA isolation or protein extraction.

K. Quantitative Real-Time Reverse Transcriptase (RT)-PCR

Approximately 10^6 β cells or 50-100 islets were lysed in 350ml of Buffer RLT (Qiagen) containing 1% β -mercaptoethanol. Samples were sheared through a 27-gauge needle, and total RNA was recovered using Qiagen RNeasy columns. Total RNA was reverse transcribed to make cDNA and subjected to qRT-PCR using SYBR Green as described previously (162).

Samples were normalized to *Actb* message levels, except in the case of RNA from polyribosomal profiling experiments, which were reported as the percent of total recovered RNA. All data represent the mean of triplicate determinations from at least three independent experiments of β cells or pooled mouse islets from three separate isolations. Primers previously described include *Bip*, *Xbp1*, *Xbp1s*, and *Chop*, *Serca2b*, *ATF4*, and *Pdx1* (41). For amplification of *Actb*, the

following primers were used: 5'- AGGTCATCACTATTGGCAACGA-3' (forward) and 5'-CACTTCATGATGGAATTGAATGTAGTT-3' (reverse). For *Nos2*, the following primers were used: 5'- CCTACCAAAGTGACCTGAAAGAGG-3' (forward) and 5'- ATTCTGTGCTGTCCCAGTGAGGAG-3' (reverse). For *Cpe*, the following primers were used: 5'- GCTCAGGTAATTGAAGTCTT-3' (forward) and 5'- TACTGCTCACGAATACAGTT-3' (reverse). For *firefly luciferase*, primers 5'- AGAGGATGGAACCGCTGGAGAG-3' (forward) and 5'- GCTTCTGCCAACCGAACGGAC-3' (reverse) were used. For *renilla luciferase*, primers 5'- CAAAGAGAAAGGTGAAGTTCGTCG-3' (forward) and 5'- TGGAAGAATCCTGGGTCCG-3' (reverse) were used.

L. Statistics

All data are presented as the mean \pm SEM. One-way ANOVA (followed by a Dunnett's post test) was used for comparisons in which two or more conditions were compared with a single control, and a Student's t test was performed where one condition was compared with a single control (using Bonferonni post test). Prism software version 5.0 (GraphPad) was used for all statistical analyses. Statistical significance was defined as $P < 0.05$.

CHAPTER 3

Maintenance of Pdx1 mRNA Translation in Islet β Cells During the Unfolded Protein Response

In this chapter, I explore changes to the translational status of islet β cells in response to known ER stress and UPR inducing agents, including proinflammatory cytokines. Particular emphasis is placed on investigating regulation of translation initiation in the islet β cell stress response. Experiments using palmitate were carried out in collaboration with Dr. Masayuki Hatanaka at Indiana University School of Medicine.

3.1 INTRODUCTION

As discussed previously, pancreatic β cells are exposed to proinflammatory cytokines in the setting of both T1D and T2D, causing β cell dysfunction and death (see Chapter 1.3D) (55,60). Little is currently known about how translational responses may contribute to such β cell failure. To study mRNA translation responses as seen in diabetes, I first established physiologic cell culture conditions that mimic the UPR. Previously, it was reported that early UPR is observed upon incubation of β cells and islets with a mixture of proinflammatory cytokines for 24h (89,90). Here, I investigate cytokine induced translational changes in islet β cells, and compare these changes to overt ER stress. In addition to assessment of general β cell translational status, I explore translational regulation of *Atf4*, *Chop*, *Cpe*, and *Pdx1* in β cells exposed to known

UPR inducers. Finally, I assess the ability of the *Pdx1* 5' UTR to mediate stress resistant translation. As a positive control for ER stress, cells were treated with thapsigargin (an inhibitor of the SERCA Ca²⁺ pump) for 4h.

3.2 RESULTS

A. Proinflammatory cytokines activate the UPR in MIN6 β cells and mouse islets

As shown in Fig. 8A, treatment of both MIN6 cells and mouse islets with thapsigargin led to clear increases in the ER stress markers *Atf4*, *Chop*, and spliced *Xbp1*, with increases in corresponding proteins levels of ATF4 and CHOP (Fig. 8B). In contrast to the overt effect of thapsigargin, treatment of MIN6 cells with cytokines for 24h led to significant increases in spliced *Xbp1* and *Chop*, but not *Atf4* mRNA, with similar findings in mouse islets. Despite the minimal changes to *Atf4* mRNA seen with cytokine incubation, I observed a significant increase in the protein levels of ATF4 in both MIN6 cells and islets (but not CHOP) (Fig. 8B), consistent with the known effect of the UPR to enhance *Atf4* mRNA translation (177). I next examined the mRNA and protein levels of factors thought to be suppressed by the UPR. The levels of the mRNA encoding the insulin processing enzyme carboxypeptidase E (Cpe) were unchanged in MIN6 cells and islets (Fig. 8A) at 24 h cytokine incubation, yet its protein levels were reduced (Fig. 8B), consistent with the known UPR-mediated suppression of this protein (178). Together, these data indicate proinflammatory cytokines induce ER stress and the appropriate UPR in MIN6 β cells and mouse islets.

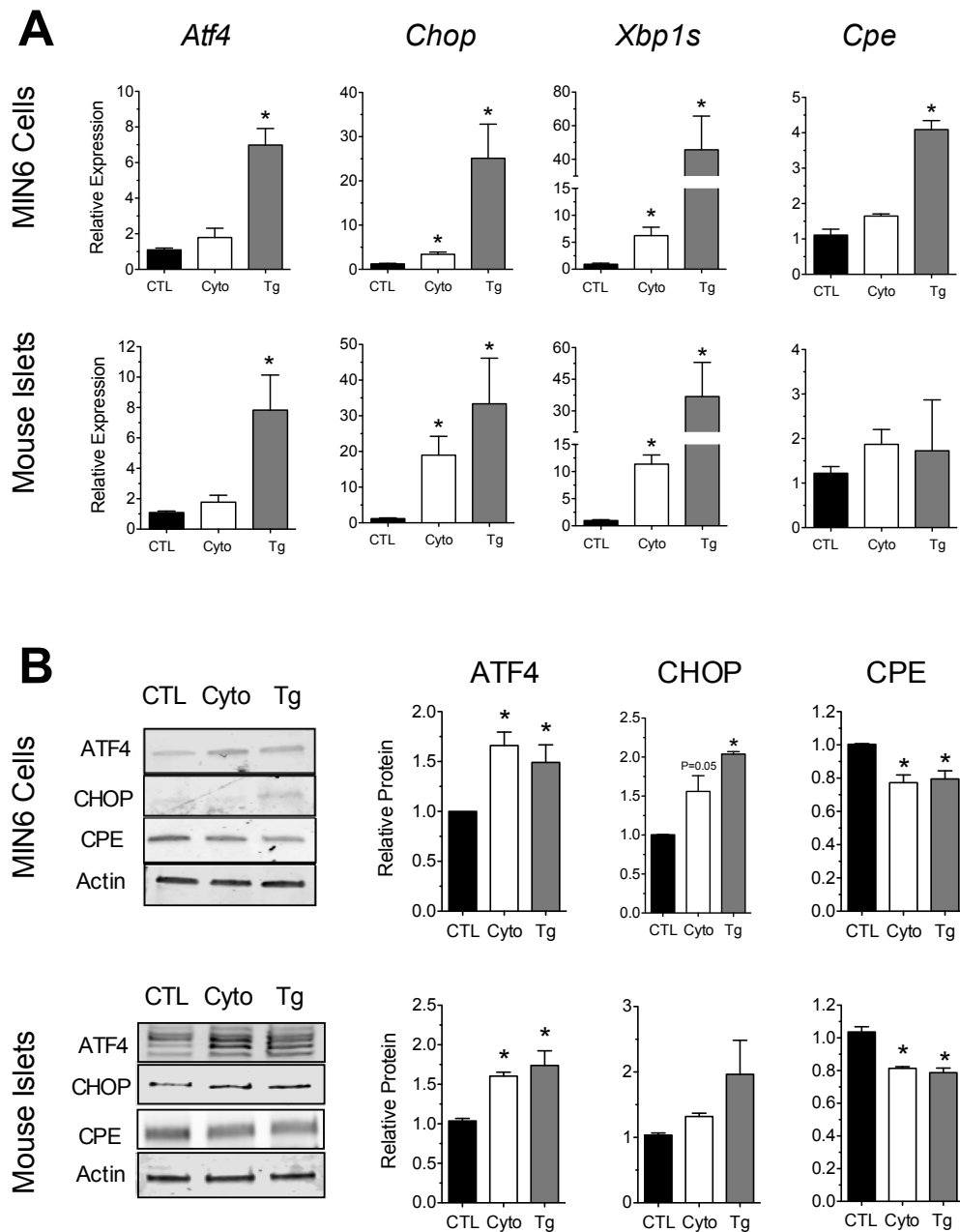


Figure 8: Proinflammatory cytokines activate the UPR in mouse MIN6 cells and islets. A, MIN6 cells (top panel) or mouse islets (bottom panel) were untreated (CTL), treated with cytokines (5ng/ml IL- β , 10ng/ml TNF- α , 100ng/ml IFN- γ) for 24h, or with 1 μ M thapsigargin (Tg) for 4h. cDNA from total cell lysates were subjected to qRT-PCR for the genes indicated. B, total cell lysates from MIN6 cells (top panel) or mouse islets (bottom panel) were subjected to immunoblot analysis following 24h cytokines or 4h thapsigargin treatment, and results were quantified and displayed in bar graphs. Data represent means \pm SEM. n=3-5. *P<0.05 compared to CTL.

B. Early effects of proinflammatory cytokines on translational regulation in MIN6

In the setting of ER stress, the UPR attempts to mitigate new protein production in part via inhibition of translation. The phosphorylation of eIF2 α by PERK leads to a block in the initiation of new mRNA translation (179). To assess the effects of cytokines and thapsigargin on mRNA translation, I subjected MIN6 cells and islets to polyribosomal profile (PRP) experiments, as outlined in Fig. 9 (180). Briefly, lysates were added atop a 10-50% sucrose gradient and sedimented by ultracentrifugation. Sedimented gradients were then subjected to measurement of RNA absorbance, and fractions were collected to assess changes to ribosomal occupancy of specific RNAs (Fig. 9). As shown in Fig. 10A and B (and in agreement with prior studies, ref. (89)), cytokine treatment of MIN6 cells and islets led to an increase in eIF2 α phosphorylation (similar, but slightly lower in magnitude compared to thapsigargin). Fig. 10A shows typical PRPs for MIN6 cells treated with and without thapsigargin, showing the positions of the 80S monoribosome-associated (initiating or inactively translating) RNAs and the polyribosome-associated (actively translating) RNAs. Compared to untreated cells, thapsigargin treatment resulted in the relative depletion of polyribosome-associated RNA compared to monoribosome-associated RNA (reflected as a reduced polyribosome/monoribosome—P/M—ratio of 0.892 ± 0.18 vs. 0.547 ± 0.1 , $P < 0.05$). This finding is consistent with the known effects of ER stress, as a block in translation initiation causes retention of monoribosomes as polyribosomes elongate and run off of RNA transcripts (180). By contrast, the

PRP of primary islets (Fig. 10B) shows general predominance of 80S monoribosomes, with little change in the P/M ratio following 24 h of thapsigargin treatment (P/M ratio of 0.999 ± 0.15 vs. 0.819 ± 0.03 , $P=NS$). Upon treatment with cytokines for 24h, neither MIN6 cells nor islets showed change in the P/M ratio. Overall, these data indicate that the early effect of cytokines to activate the UPR is not evident from examination of the PRP.

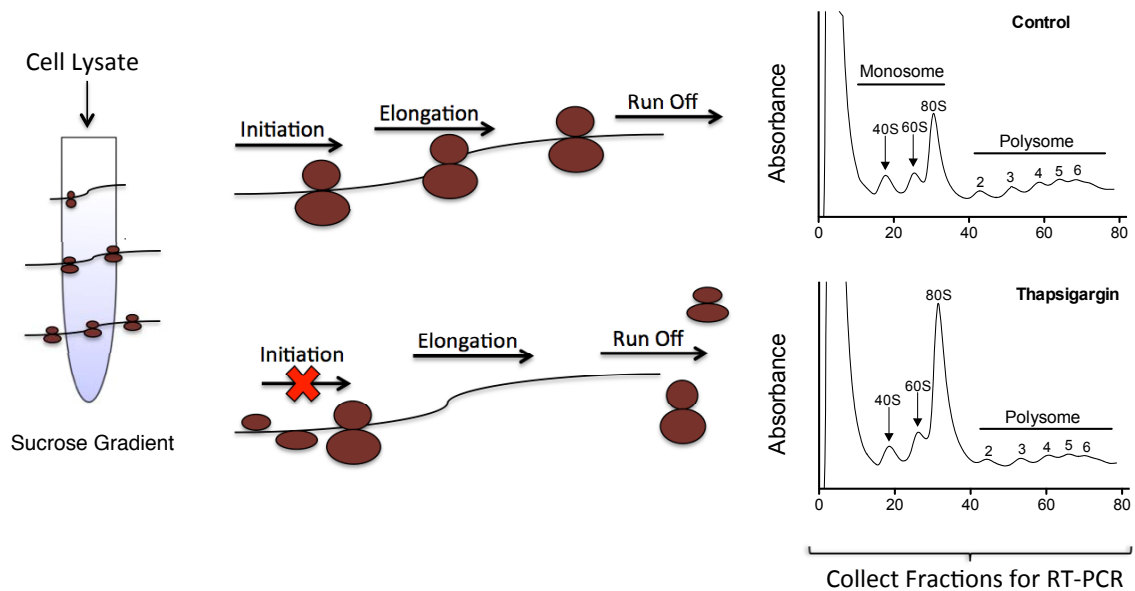


Figure 9: Polyribosomal profiling (PRP) reveals changes to ribosomal occupancy of RNA. Polyribosomal profiling (PRP) facilitates analysis of translation via assessment of ribosomal occupancy of total RNA as well as specific RNAs. Cell or islet total cell lysates were added atop a 10-50% sucrose gradient, and sedimented at 270,000g for 2h. A piston gradient fractionator was used to fractionate the gradient, while an in-line UV monitor measured RNA absorbance at 254nm. This data indicates the general translational status of β cells, as measured by ratio of polyribosome associated RNA. Ten fractions per gradient were collected, and total RNA was isolated, reverse transcribed, and subjected to qRT-PCR for each fraction. This data indicates how ribosomal occupancy of specific transcripts changes in response to stress.

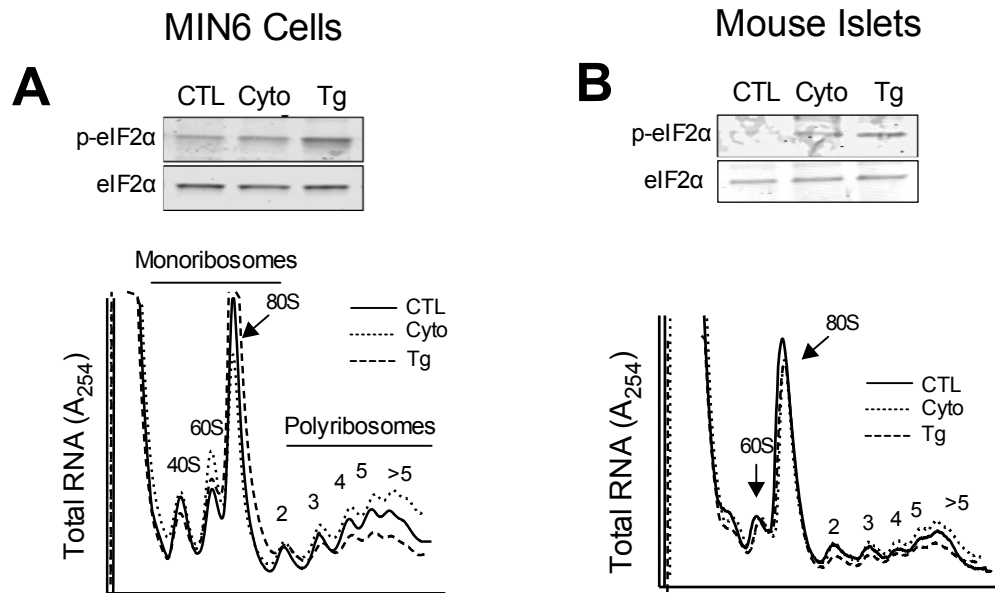


Figure 10: Early effects of proinflammatory cytokines on translational regulation in MIN6 β cells and mouse islets. A, MIN6 β cells were untreated (CTL), treated with cytokines (Cyto) for 24h, or treated with 1 μ M thapsigargin (Tg) for 4h, then subjected to immunoblot analysis for eIF2 α phosphorylation (top panel), or PRP analysis (bottom panel); B, as in panel A, with mouse islets. Representative data from 3 independent experiments are shown for each.

C. Prolonged exposure to proinflammatory cytokines induces an apparent block in translational initiation

ER stress triggers the PERK-mediated phosphorylation of eIF2 α , causing a block in the translational initiation of most cellular mRNAs in an attempt to decrease ER protein load (179,181). Because cytokine release from adipocytes and from infiltrating immune cells is believed to contribute to islet dysfunction and reduced insulin release from islets in type 2 and type 1 diabetes respectively, I asked whether prolonged exposure of cells to cytokines causes a translational initiation block, consistent with the ER stress response. To assess the translational effects directly, I performed PRP. Fig. 11A shows the positions of the 40S, 60S, and 80S ribosomal species, as well as polyribosomes (which contain multiple ribosomes bound to individual transcripts) from the total RNA of control MIN6 cells. The ratio of polyribosomes to 80S monoribosomes (P/M ratio) is 1.59 in these control cells. A 4-hour treatment of MIN6 cells with 1 μ M thapsigargin (a SERCA pump inhibitor and inducer of ER stress) results in the dissipation of the polyribosome fraction and a decrease in the P/M ratio to 0.85, consistent with a block in initiation and a resultant runoff of polyribosomes (Fig. 11A). This ribosomal runoff and fall in the P/M ratio is considered a hallmark of translation initiation blockade (179). During a timecourse of treatment of MIN6 cells with a cocktail of cytokines (IL1- β , TNF- α , and IFN- γ), there was a gradual loss of the polyribosomal fraction and a decrease in the P/M ratio that occurred after 24 hours (Fig. 11B). To verify that this loss of polyribosomes resulted in a decrease in total protein synthesis, I incubated MIN6 cells with cytokines for 0,

24, or 72 hours, then with a mixture of ^{35}S -Met and ^{35}S -Cys for 1 h. As shown in Fig. 11C, after loading correction for total cellular uptake of ^{35}S , cytokine treatment resulted in a global decrease in total ^{35}S incorporation into protein at 72h. Further, blockade of translation initiation in this model is not attributable to the effects of iNOS, as co-incubation with the NOS inhibitor L-NMMA (1mM) did not result in retention of polyribosome associated mRNA (Fig. 11D). These data demonstrate that prolonged proinflammatory cytokine treatment results in a decrease in protein synthesis and polyribosome associated RNAs, consistent with blockade of translation initiation and ER stress.

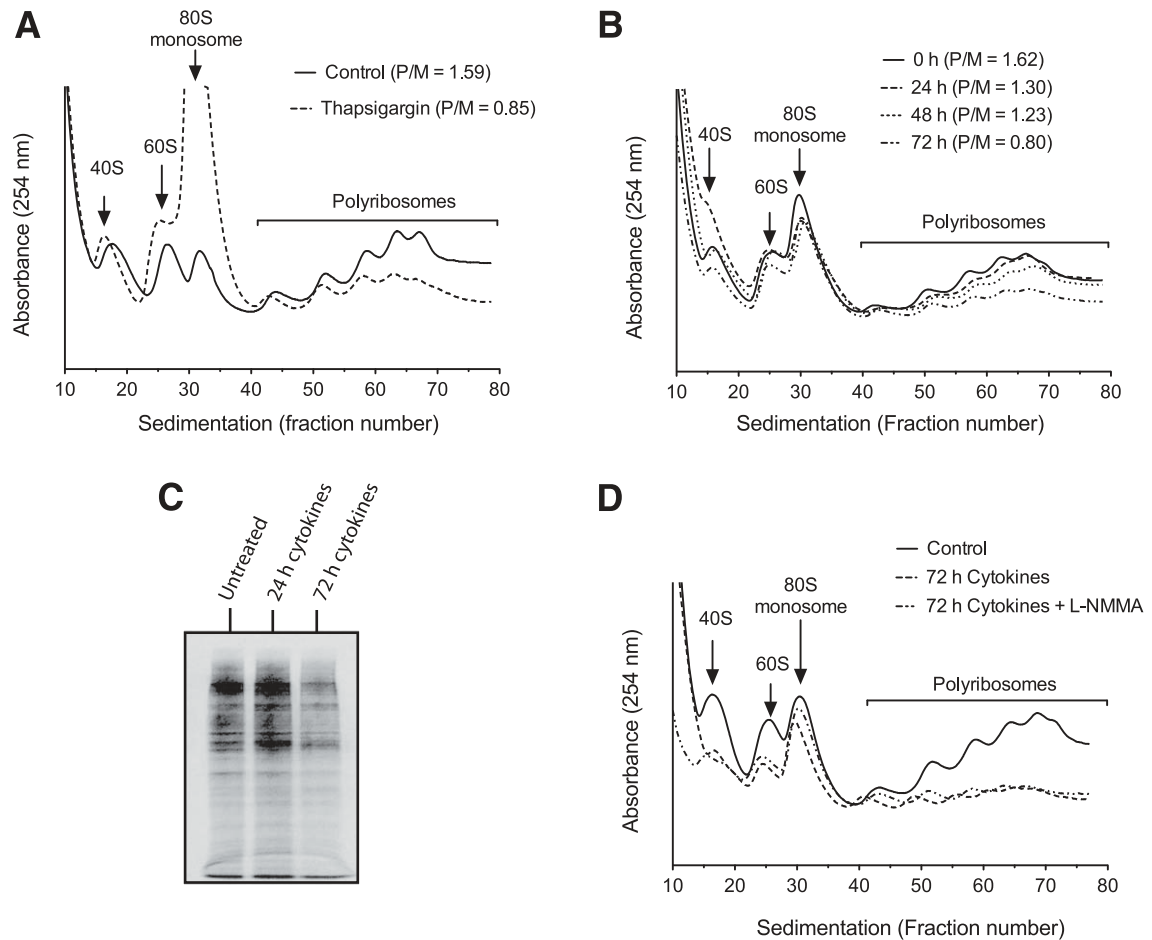


Figure 11: Prolonged exposure to proinflammatory cytokines induces an apparent block in translational initiation. MIN6 cells were treated with $1\mu\text{M}$ thapsigargin, or cytokines for the indicated times, and subjected to PRP or ^{35}S -Cys/Met incorporation. A, polyribosomal profiles of MIN6 cells untreated or treated with thapsigargin; B, profiles of MIN6 cells treated with cytokines for the indicated times; polyribosome to monoribosome (P/M) ratios are indicated for each condition; C, proteins labeled with ^{35}S -Cys/Met for 1 hour after cytokine treatment were subjected to SDS-PAGE analysis to compare total protein synthesis; D, MIN6 cells were coincubated with cytokines and the NOS inhibitor L-NMMA for the indicated times, and subjected to PRP. Representative data from 3 independent experiments are shown for each.

D. Chronic palmitate incubation increases markers of the UPR and reduces polyribosome-associated RNAs in β cells

In addition to proinflammatory cytokines, we undertook experiments to determine the effects of saturated free fatty acids on β cell translation. Saturated FFAs have been shown to activate the unfolded protein response (UPR), leading to ER stress and β cell dysfunction (2). As shown in Fig. 12A, mRNA markers of UPR activation including spliced *Xbp1*, *Bip*, *Atf44*, and *Chop* are increased at 72h after palmitate incubation in MIN6 cells (at levels at or below those seen with 4h of thapsigargin incubation). To study the effects of saturated FFAs on mRNA translation in β cells, we incubated MIN6 β cells with 0.5mM palmitate and subsequently performed PRP analysis. The normal PRP of MIN6 β cells is shown in Fig. 12B, and is identified with a solid line. After 72h of palmitate addition, there was a decrease in the fraction of RNAs associated with polyribosomes (Fig. 12B, dashed line), which was reflected by a decreased P/M ratio (Fig. 12B). As shown in Fig. 12C, palmitate incubation of MIN6 cells led to increases in phosphorylated eIF2 α with time. Incubation of MIN6 β cells for 4 h with 1 μ M thapsigargin, a very potent inducer of phosphorylated eIF2 α and the UPR, resulted in a similar reduction in the P/M ratio (Fig. 12D), accompanied by increases in phosphorylated eIF2 α (Fig. 12E). The results in Fig. 12 collectively indicate that palmitate causes a progressive reduction in global translation initiation that is consistent with activation of the UPR.

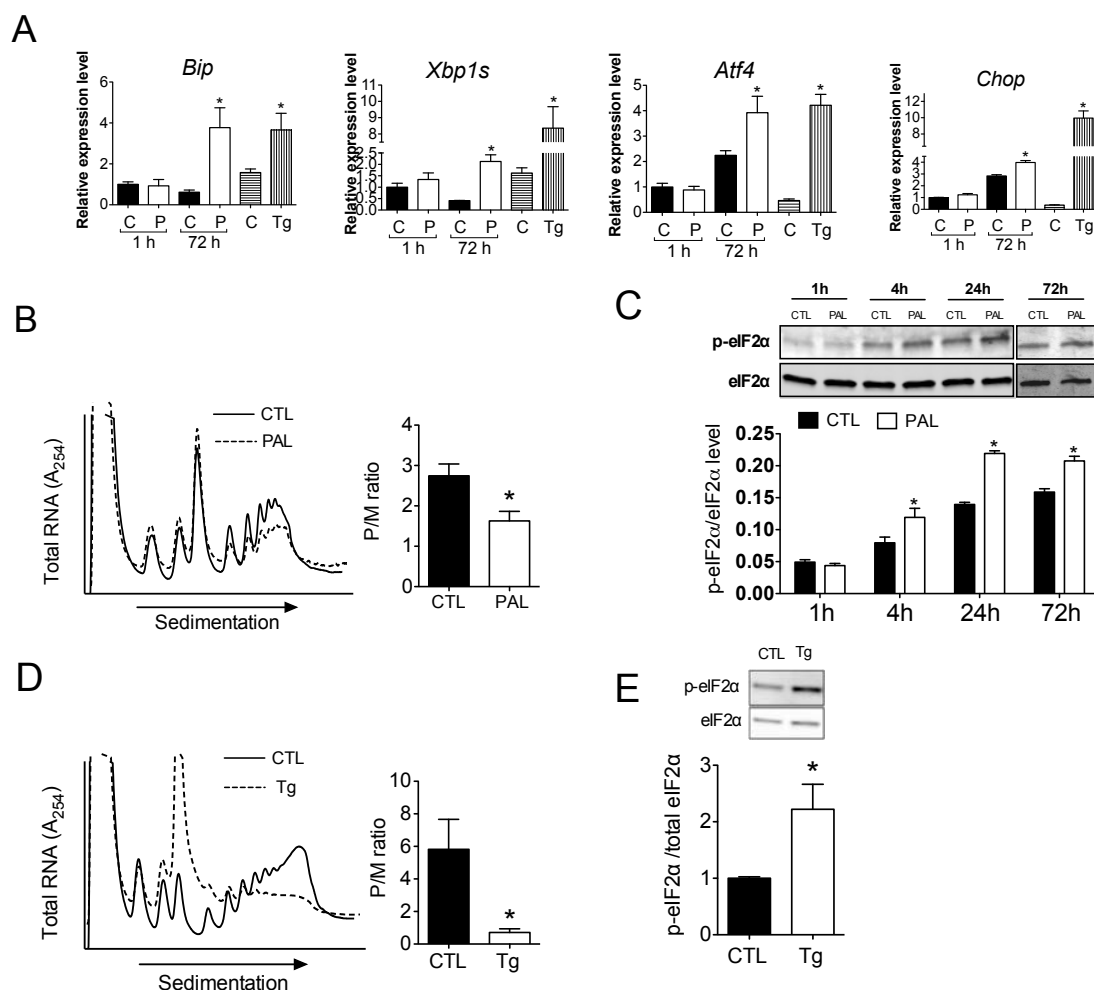


Figure 12: Chronic palmitate incubation increases markers of the UPR and reduces polyribosome-associated RNAs in β cells. A, MIN6 β cells were untreated, treated with palmitate for 1h or 72h, or treated with thapsigargin for 4h, and cDNA from total cell lysates were subjected to qRT-PCR for the indicated genes; MIN6 β cells were treated with palmitate for 72h (B) or with thapsigargin for 4h (D), then subjected to PRP analysis for P/M ratio quantification; MIN6 cells were treated with palmitate for the indicated times (C) or with thapsigargin for 4h (E), then subjected to immunoblot analysis for quantification of phospho-eIF2 α . Data represent means \pm SEM. $n \geq 3$. * $P < 0.05$ compared to CTL.

E. Proinflammatory cytokines activate *Atf4* and *Chop* translation, and repress *Cpe* translation in MIN6 β cells

In our cytokine and FFA treated MIN6 models of translational regulation, we observed elevated eIF2 α phosphorylation prior to loss of polyribosome associated RNAs. I hypothesized that the PRPs (which reflect total RNA engagement at ribosomes) may be relatively insensitive to the translational events induced by stressors at early time points, and that alterations in engagement of specific mRNAs with polyribosomes may be more evident. To examine specific mRNA engagement with mono- and polyribosomes, I next performed real-time qRT-PCR from individual PRP fractions to quantitate changes in ribosome association. I found that *Atf4* mRNA was shifted rightward toward greater occupancy by polyribosomes following proinflammatory cytokine treatment—similar to thapsigargin treatment—suggesting increased ribosome engagement and mRNA translation under these conditions (Fig. 13A). This shift towards polyribosomes is in agreement with the increased protein levels of ATF4 observed under ER stress conditions (Fig. 8B). Similarly, I observed a rightward shift of *Chop* mRNA towards polyribosomes with cytokine and thapsigargin treatments (Fig. 13B). These data suggest that the established mechanism of phospho-eIF2n mediated translational activation of *Atf4* and *Chop* via uORF is conserved in the islet β cell. In contrast, *Cpe* mRNA showed a leftward shift into monoribosomes under both cytokine and thapsigargin conditions (Fig. 13C), consistent with reduced engagement of translating ribosomes and reduced carboxypeptidase E levels (Fig. 8B). This data supports recent evidence that

Cpe translation is stress sensitive and is regulated by a cap-dependent mechanism (132). These data suggest that proinflammatory cytokines, similar to thapsigargin, induce differential effects on mRNA translation depending upon the nature of the transcript.

F. Palmitate activates *Atf4* translation in MIN6 β cells

A classic feature of saturated FFA induced β cell dysfunction is activation of the UPR. Consistent with this idea, 24h palmitate treatment of MIN6 β cells revealed a shift of *Atf4* mRNA from monoribosome fractions to polyribosome fractions (Fig. 14A). This change is comparable to that seen with thapsigargin treatment, and indicates a palmitate induced translational activation of *Atf4* (Fig. 14B).

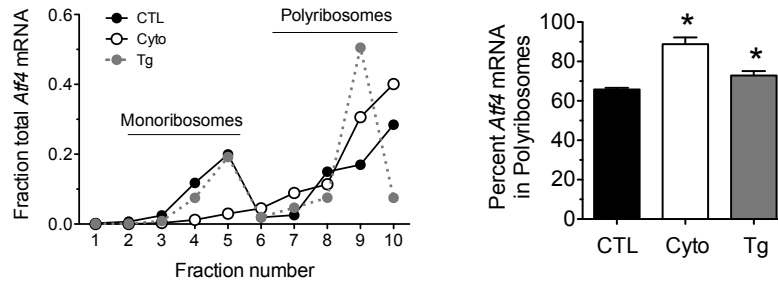
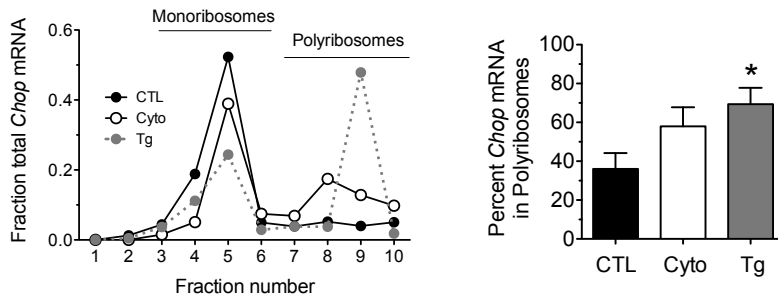
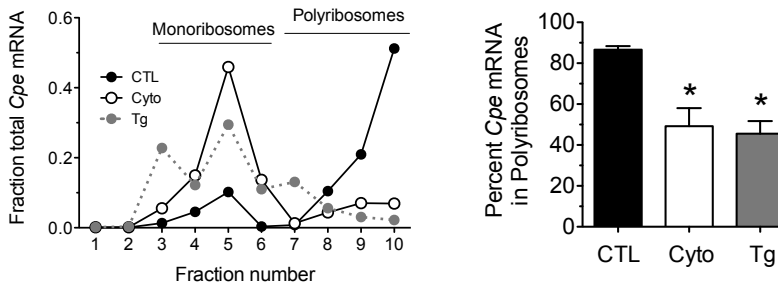
A**B****C**

Figure 13: Proinflammatory cytokines activate *Atf4* and *Chop* translation, and repress *Cpe* translation MIN6 β cells. MIN6 β cells were untreated (CTL), treated with cytokines for 24h (Cyto), or with thapsigargin for 4h (Tg), and subjected to PRP analysis with fractionation of the sedimentation gradient; A, analysis of *Atf4* mRNA in PRP fractions; B, analysis of *Chop* mRNA in PRP fractions; C, analysis of *Cpe* mRNA in PRP fractions. Representative data are shown on the left of panel A, B, and C, and the percent of total mRNA in polyribosomes is quantified on the right. Data represent mean \pm SEM. $n=3-5$. * $P<0.05$ compared to CTL.

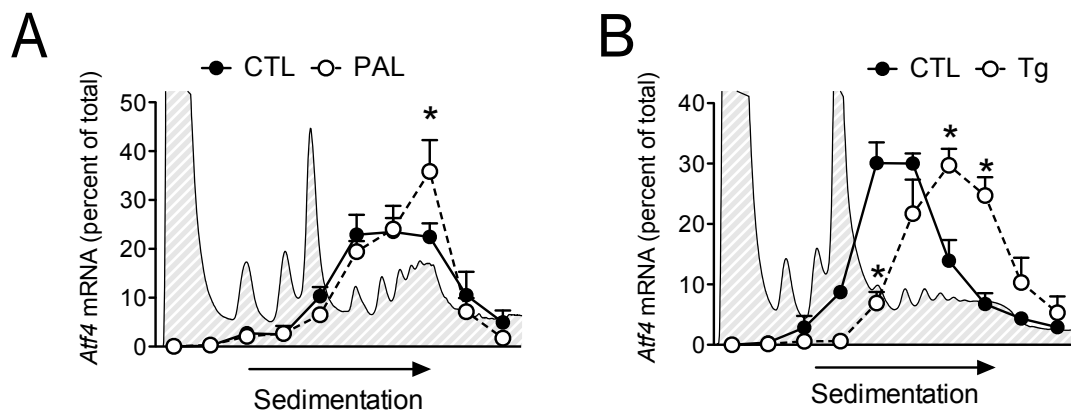


Figure 14: Palmitate activates *Atf4* mRNA translation in MIN6 β cells. MIN6 β cells were treated with palmitate for 72h (A), or with thapsigargin for 4h (B), and subjected to PRP analysis with fractionation of the sedimentation gradient; A, analysis of *Atf4* mRNA in PRP fractions following palmitate treatment (open circles); B, analysis of *Atf4* mRNA in PRP fractions following thapsigargin treatment (open circles). Data represent means \pm SEM. $n \geq 3$. * $P < 0.05$ compared to CTL.

G. *Pdx1* mRNA maintains ribosomal occupancy in the setting of the UPR

Pdx1 was recently shown to activate genes necessary for the adaptive UPR. Thus, I hypothesized that *Pdx1* mRNA translation must be maintained as a requisite for adaptation to ER stress (32). As shown in Fig. 15A and B, neither *Pdx1* mRNA nor *Pdx1* protein levels changed following 24h treatment with cytokines or 4h treatment with thapsigargin in MIN6 cells. In spite of the phosphorylation of eIF2 α under these conditions, the association of *Pdx1* mRNA with polysomes was unchanged following both cytokine and thapsigargin treatment (Fig. 15C). These data suggested to us that *Pdx1* mRNA translation was maintained by mechanisms that are distinct from those that suppress translation of *Cpe*, or that enhance translation of *Atf4* and *Chop*.

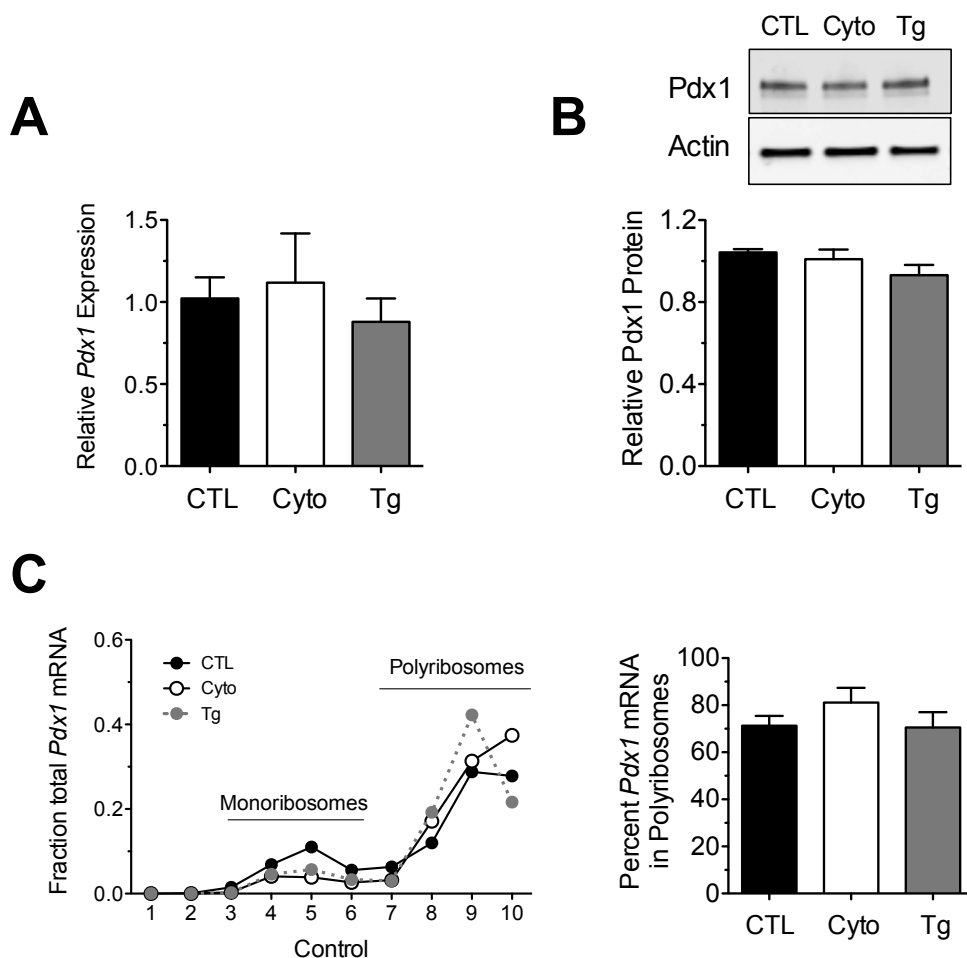


Figure 15: Pdx1 mRNA retains ribosomal occupancy in the setting of the UPR. MIN6 β cells were untreated (CTL), treated with cytokines for 24h (Cyto), or with thapsigargin for 4h (Tg), then subjected to qRT-PCR, immunoblot analysis, or PRP analysis with fractionation of the sedimentation gradient. A, qRT-PCR analysis from whole cell extracts; B, immunoblot analysis (top panel), with corresponding quantification (n=3-5, lower panel); C, qRT-PCR analysis of *Pdx1* mRNA in PRP fractions (left panel), and corresponding quantification of percent of total mRNA in polyribosomes. Data represent mean \pm SEM, n=3-6.

H. The *Pdx1* mRNA 5'-UTR enhances mRNA translation in the setting of the UPR

mRNAs containing a 5'-7 methylguanylate “cap” are targeted for eIF2 α dependent translation initiation, and are translationally repressed when eIF2 α is phosphorylated during the UPR (182). Many privileged mRNAs contain elements in the 5'-UTR that aid in ribosome recruitment and initiation under conditions of stress (see Chapter 1.6A) (182). To investigate if the maintenance of *Pdx1* mRNA translation during the UPR is governed by its 5'-UTR, I cloned the 5'-UTR of the mouse *Pdx1* gene into a bicistronic luciferase reporter vector (C49) between the renilla and firefly luciferase genes (176). This vector allows the expression of the first gene (renilla luciferase) to be driven by conventional, cap-dependent translation initiation, while expression of the second gene (firefly luciferase) is driven by cap-independent translation initiation mediated by the cloned 5'-UTR element(s) (Fig. 16A). Upon transfection of the bicistronic reporter constructs into cell lines, the ratio of the firefly:renilla luciferase activity is a gauge of intrinsic ribosome initiation activity (176).

As shown in Fig. 16B, the mouse *Pdx1* mRNA 5'-UTR showed ~15-fold enhancement of the firefly:renilla activity ratio in MIN6 cells compared to an empty vector control or to a bicistronic vector containing the Cpe 5'-UTR (Fig. 16B). Mouse *Pdx1* mRNA 5'-UTR also displayed a ~5-fold enhancement of firefly:renilla activity ratio compared to the human surfactant protein A (hSPA) 5'-UTR, which contains a known internal ribosomal entry site (176). To determine if the human *PDX1* 5' UTR also directed cap-independent translation, I inserted the

human PDX1 5'-UTR into the bicistronic reporter vector. The human PDX1 5'-UTR showed a ~65-fold enhancement of the firefly:renilla luciferase activity ratio in MIN6 β cells compared to empty vector control (Fig. 16B). Importantly, the increased firefly:renilla activity ratios with the human and mouse 5'-UTRs were not caused by a cryptic promoter element, because ratios of firefly:renilla luciferase mRNAs by real-time PCR were identical for all constructs tested (Fig. 16C). Next, I carried out these experiments under cytokine and thapsigargin treated conditions to determine if translational enhancement persists under stress conditions. As shown in Fig. 17, the Pdx1 5'-UTR continues to significantly drive firefly:renilla activity at a level comparable to that of hSPA, which contains an internal ribosomal entry site. However, the firefly:renilla activity ratio was reduced approximately 3 fold under conditions of cytokine stress (Fig. 16B and Fig. 17A). Collectively, the data in Figures 15-17 indicate that Pdx1 5'-UTR drives cap-independent translation of downstream ORFs and that its activity is comparable to that of known internal ribosomal entry sites under conditions of ER stress.

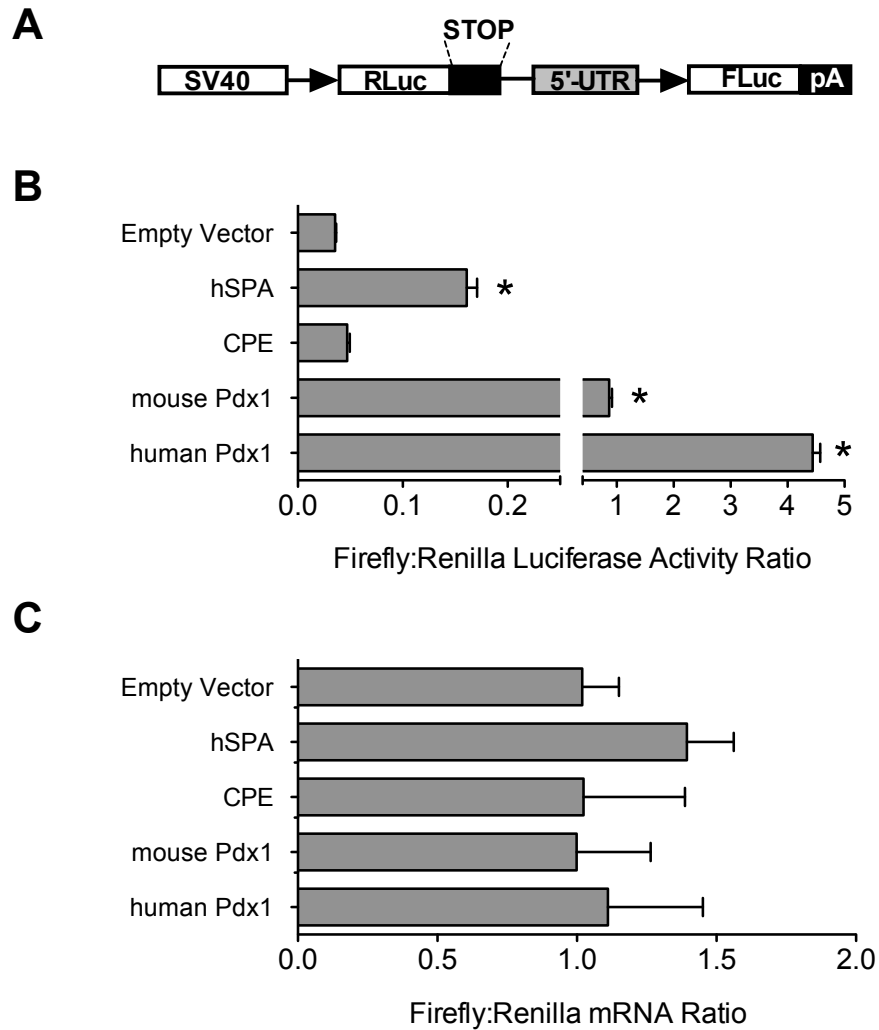


Figure 16: Pdx1 5' UTR allows cap-independent translation initiation of downstream ORFs. A, schematic diagram of the bicistronic reporter vector, showing positions of the SV40 promoter, renilla luciferase gene (RLuc), 5' UTR, and firefly luciferase gene (FLuc); B, MIN6 cells were transfected with bicistronic vector containing no insert (empty vector), 5' UTR of hSPA, 5'UTR of Cpe, and 5'UTRs of mouse and human Pdx1 genes, then subjected to dual luciferase assay; C, same as in panel B, except that cDNA from each condition was subjected to qRT-PCR for firefly and renilla mRNAs (normalized to Actb mRNA). Data represent mean \pm SEM. $n=3-8$. * $P<0.05$ compared to empty vector.

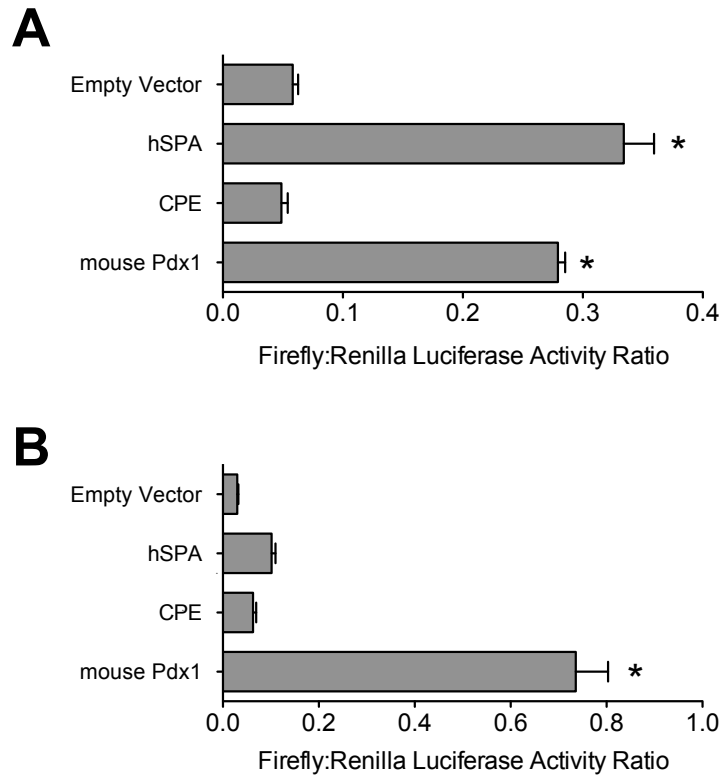


Figure 17: Pdx1 5' UTR facilitates cap-independent translation in the setting of the UPR. MIN6 β cells were transfected with the bicistronic reporter vectors containing no insert (empty vector), *hSPA*, *Cpe*, or mouse *Pdx1* 5'UTRs, then treated with cytokines for 24h, or thapsigargin for 4h, and subjected to dual luciferase assay. A, results after cytokine treatment; B, results after thapsigargin treatment. Data represent mean \pm SEM. $n=3-8$. * $P<0.05$ compared to empty vector.

I. Upstream open reading frames (uORFs) in the *Pdx1* 5'-UTR are not required for enhanced mRNA translation

Recent studies suggest that upstream open reading frames (uORFs) in the 5' UTR may play permissive or inhibitory roles in the translation of certain physiologic downstream ORFs (142). As shown in Fig. 18A, comparison of mouse and human *Pdx1* 5'-UTRs revealed 70% identity and the existence of a conserved uORF beginning at bp –171(mouse) and at bp –187 (human) relative to the protein coding translation start site. Deletion analysis of the *Pdx1* 5' UTR was carried out using the constructs outlined in Fig. 18B. Deletion of the proximal region of the mouse 5' UTR, including a proximal portion of the uORF (deletion mutant 1 - DM1), did not affect reporter activity, leading us to hypothesize that translation of this uORF is not required for enhanced translation of the coding ORF (Fig. 18C). Conversely, deletion of the distal region of the 5' UTR (containing the uORF start site, deletion mutant 2 - DM2) led to complete loss of the ability of the element to enhance translation of firefly luciferase in both the mouse and human element (Fig. 18C), suggesting that the region –105 to –280 contains elements that promote translation. Notably, these alterations in firefly:renilla activity ratios were not caused by changes in firefly:renilla message (Fig. 18D). Together, these data indicate that elements in the distal portion of the *Pdx1* mRNA 5' UTR are required for full activity of this translation enhancing element.

A

```

-300
Human  - - - - - GCA GGT Gc TCGCGGGt ACCT g g GCCT a GC c TCTTA g t GC g Gc Cag
Mouse  AAAA TTGAAA CAAGT GCAGGTGt TCGCGGGc ACCT aa GCCT c - Ct TCTTA ag GCa Gt Cc t

-240
Human  CCA GGCCAA T c A c GGC c CC c GG c Tg AA CCA CGT GGGG c c CC g Cg GAG CCT A T GG t g CGGC
Mouse  CCA GGCCAA T g A t GGC t CC a GG T a AA CCA CGT GGGG t g CC c Ca GAG CCT A T GG c a CGGC

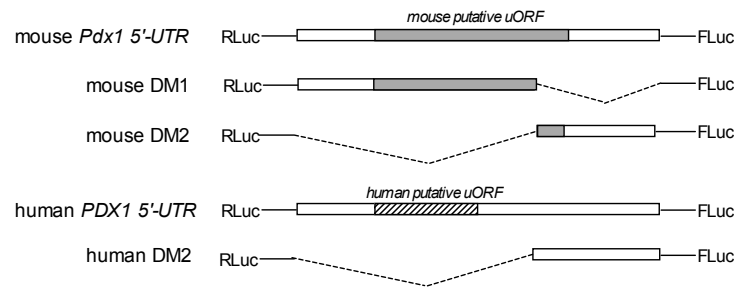
-180
Human  GGCCGGC c c Gc CGGT CC g c GC t g GC t g t g GGT TCC c t c t GAGA t CAG TG c g GAg CT GT CA
Mouse  GGCCGGC t t Gt C - - CC c a GC c a GC c t c t GGT TCC c a g GAGA g CAG TG g a GAg a CT GT CA

-120
Human  AAG CGA g Ca GGGGT GGC G c c Gg GAG T g g Gaa c GCC AC a CAG t GCC a AA t c CC c GGCT c - c
Mouse  AAG CGA t Ct GGGGT GGC G t a GAG T c c Gc g a GCC AC c CAG c GCCT AA g g CC t GGCT t Gt

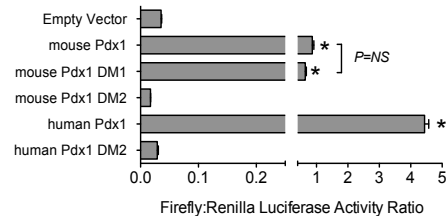
-60
Human  AGCT c - CC GA c t C c c GGCT c C c GGCT CCC g g c T c CCGG t g c CCA a t CCCGGG CCGCAG CC
Mouse  AGCT m R CCGA C c C g g GGCT g Ct GGC c CCC a a g T g CCGG c t g CCA c c - - - - -

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B



C



D

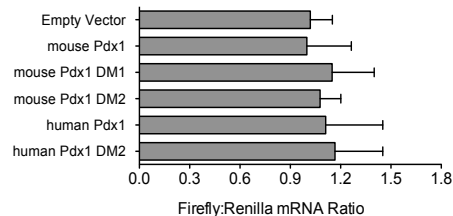


Figure 18: Deletional analysis of the Pdx1 5' UTR. A, line-up of mouse and human *Pdx1* 5' UTRs, showing in gray the poitions of putative uORFs; B, schematic representation of deletion mutants of the mouse and human *Pdx1* 5' UTR that were inserted into the bicistronic vector between the renilla luciferase (RLuc) and firefly luciferase (FLuc) genes; C, results of dual luciferase assays following transfection of the constructs in panel B into MIN6 β cells; D, qRT-PCR from total RNA for renilla luciferase and firefly luciferase mRNAs (normalized to *Actb* mRNA). Data represent mean \pm SEM. $n=3-8$. * $P<0.05$ compared to empty vector.

3.3 DISCUSSION

In T1D, protein unfolding and ER stress in the β cell is thought to arise when local or generalized inflammation (arising from innate or adaptive immune processes) triggers pathways that lead to nitric oxide production, oxidative stress, and/or reductions in SERCA2 levels (183). In T2D, proinflammatory cytokines, free fatty acids, and insulin resistance disrupt β cell ER homeostasis. Under these conditions, the UPR induces generalized reductions in mRNA translation initiation, along with a simultaneous increase in translation of specific mRNAs needed for stress remediation (182). In these studies, I examined the differential β cell translational responses to known ER stressors. I observed changes to ribosomal occupancy of total cell RNA, as well as to key stress-responsive mRNAs in the β cell.

The translational regulation of mRNAs occurs predominately at the level of translation initiation, where factors including eIF2 α , eIF4E, and the eIF4F complex control initiation (137,150). Under conditions of fuel excess, pathways such as the mammalian target of rapamycin (mTOR) promote eIF4E activity to enhance translation initiation (184). By contrast, under conditions of stress, such as during fuel (amino acid) deficiency or ER stress, translation of many 5'-7-methylguanylate-“capped” transcripts is repressed at the level of initiation (182). This repression is mediated by eIF2 α phosphorylation and availability and assembly of cap binding translation factors. In the β cell, it was recently shown that translation of mouse *Cpe* mRNA is dependent upon the cap component eIF4G1 for continued translation, and is subject to translational repression under

conditions of ER stress (132). By contrast, other mRNAs are translationally activated during stress. *Atf4* and *Chop* mRNA each contain inhibitory upstream open reading frames (uORFs) that repress translation of the downstream coding ORF under normal conditions. Under conditions of ER stress, however, initiation at these uORFs is repressed, while translation from the coding ORF predominates (127,185).

Our studies demonstrate that both activation and repression of translation initiation is pertinent to β cell physiology under ER stress, and that physiological stressors including proinflammatory cytokines and free fatty acids are sufficient to induce such translational control. With respect to global β cell translation, chronic exposure of β cells to proinflammatory cytokines resulted in loss of polyribosome associated RNAs, suggestive of a translation initiation block consistent with ER stress (Fig. 10-11). Interestingly, whereas thapsigargin causes rapid shut-down of global translation initiation (hours), I did not observe this until after prolonged cytokine treatment (days), indicating important differences in the effects of thapsigargin and cytokines on β cell translation.

In addition to causing a rapid shut down of general translation initiation in β cells, thapsigargin concurrently activates translation of *Atf4* and *Chop* in MIN6 β cells, increasing expression of these proteins. I next examined translational activation of *Atf4* and *Chop* in response to proinflammatory cytokine treatment. In contrast to thapsigargin treatment, I observed cytokine-induced changes to ribosomal occupancy and protein expression of *Atf4*, *Chop*, and *Cpe*, prior to changes in global translation as assessed by PRP analysis. These findings

indicate that physiological stressors such as cytokines induce translational regulation of specific transcripts via eIF2 α phosphorylation prior to a global shut down of translation initiation. Such responses may be critical to an appropriate β cell stress response.

These studies are among the first to examine translation in the islet β cell using techniques to measure ribosomal occupancy of total RNA as well as specific RNAs under conditions of diabetes relevant stressors. They demonstrate that physiological models of ER stress result in global translation initiation shut down, as well as translational activation of specific stress remedial transcripts. Interestingly, changes to ribosomal occupancy of specific messages is evident prior to general translation shut down in the case of cytokine treatment, suggesting early activation of important stress remedial translational mechanisms in the setting of diabetes.

A key objective of these studies was to investigate mechanisms regulating Pdx1 translation in the islet β cell. Pdx1 is a protein of critical importance to the β cell, as it is necessary for β cell development and for the normal function of mature β cells. It has also been shown to play a key role in β cell ER stress remediation, as it appears to function as a transcriptional activator of genes important for ER homeostasis including *Atf4*, *Wfs1*, and *Ero1b*, and haploinsufficiency of Pdx1 in mice predisposes animals to ER stress and β cell apoptosis (13,32). Previously, we showed that in the setting of type 1 diabetes in the NOD mouse, failure to maintain Pdx1 protein levels in the β cell may be a

contributing factor to the increasing ER stress and β cell dysfunction seen in that model (41).

Our studies here are the first to provide important insight into a potential mechanism whereby Pdx1 mRNA translation is maintained at elevated levels during ER stress despite global suppression of translation initiation. Our studies identified that a discrete region of the 5'-UTR of the mouse Pdx1 mRNA has properties that enhance translation of downstream coding regions. Using a bicistronic reporter vector, I showed that the region between –105 and –280 is able to independently drive reporter gene translation, whereas the region between -1 and –104 and the 5'-UTR of *Cpe* are unable to do so. Importantly, the homologous regions of the human PDX1 gene retained similar properties, suggesting that the phenomenon may be applicable to humans.

The mechanisms driving translation of mRNAs during stress have been studied for a number of genes, and include such phenomena as internal ribosome entry sites (IRESs) and upstream open reading frames (uORFs). uORFs have been estimated to occur in up to 40% of transcripts (142), but whether such elements function in a regulatory manner requires experimental verification. I identified that conserved uORFs (with AUGs at positions –171 and –213) exist in the mouse and rat Pdx1 5'-UTR, with AUG occurring at position –171 being conserved with humans. Deletion of a proximal region of this uORF (DM1) did not affect reporter activity. Further, we did not observe changes to *Pdx1* ribosomal occupancy similar to that of mRNAs known to be regulated by upstream open reading frames (*Atf4*, *Chop*). These findings suggest regulatory

mechanisms other than uORFs contribute to 5' UTR mediated stress resistant translation of Pdx1.

IRESs are a heterogeneous group of elements found in the 5' UTR of viral and cellular mRNAs which allow end-independent recruitment of ribosomes for translation initiation (137,141). It is believed that secondary structure in IRESs allows recruitment of translation initiation factors and ribosomal components necessary for the internal assembly of competent ribosomes (137). IRESs have been identified as regulatory elements in other β cell mRNAs including *Bip* and *Xiap* (148,186). Interestingly, the Pdx1 5' UTR contains ~75% G-C content, and significant predicted secondary structure, suggestive of IRESs elements. Further studies are required to determine the precise mechanism of action of this regulatory element.

Taken together, our findings shed new light on mechanisms to maintain Pdx1 protein synthesis under conditions of stress, which is critical to β cell well-being. While the *Pdx1* mRNA 5' UTR confers cap-independent translation compared to controls, it is important to note this activity was reduced under conditions of stress, particularly in the case of cytokine treatment. Thus, it is feasible that failure of cap-independent *Pdx1* translation in the setting of diabetes contributes to β cell dysfunction and death. Although not directly tested in this study, it is also conceivable that regulation of *Pdx1* mRNA translation by its 5'-UTR may also be important during pancreas development since cellular differentiation might also be considered a form of cellular stress. *Pdx1* mRNA exhibits unique properties under stress that contribute to its ongoing translation.

A better understanding of the mechanisms involved in *Pdx1* translation—such as the factors that may promote *Pdx1* mRNA translation via its 5'-UTR—could lead to therapies aimed at preserving Pdx1 expression and β cell function in diabetic states (Fig. 19).

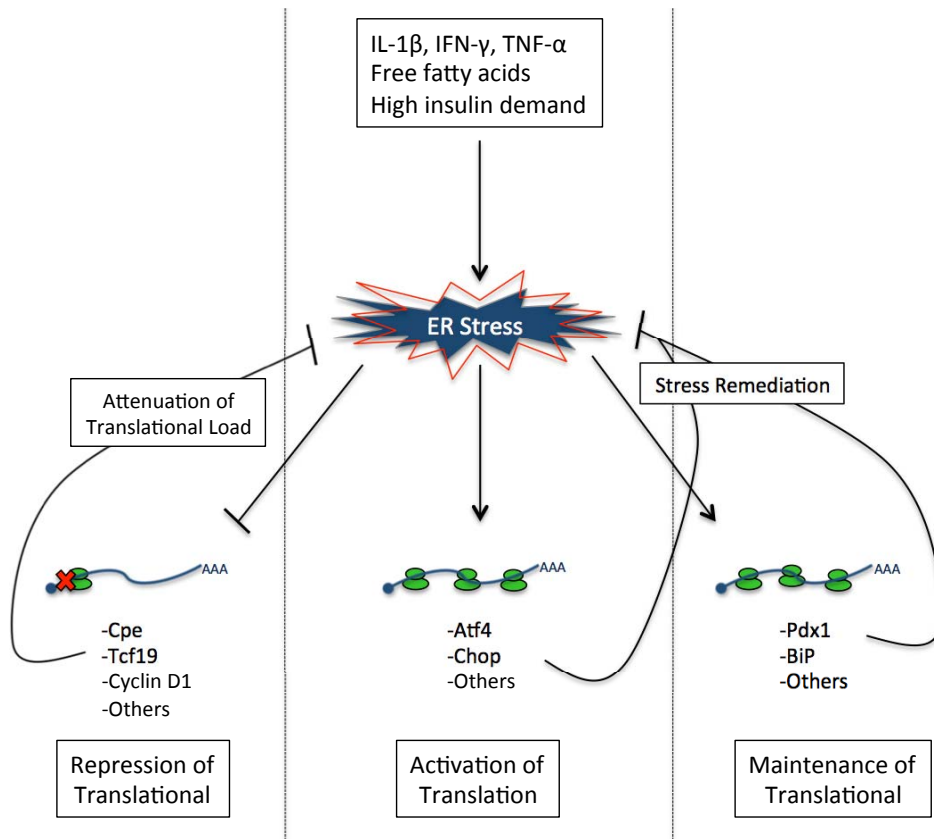


Figure 19: Regulation of translation initiation during the β cell UPR. β cell stressors including proinflammatory cytokines, free fatty acids, and high insulin demand lead to ER stress and the UPR. During this response, a new program of translational control prevails, which suppresses translation initiation of a number of transcripts, while allowing translational activation of other transcripts, while still other transcripts are translationally resistant to such stress. Together, these translational responses work to remediate ER stress and restore β cell homeostasis.

CHAPTER 4

Deoxyhypusine Synthase Haploinsufficiency Attenuates Acute Cytokine Signaling

In this chapter, I investigate the role of deoxyhypusine synthase in development, proliferation, glucose homeostasis, and translation of stress-specific β cell transcripts. Whole-body Dhps knockout mice provided a model of genetic DHS deficiency that was used to validate studies using a small molecule inhibitor of DHS. Mouse phenotyping experiments were done in collaboration with Dr. Sarah Tersey at Indiana University School of Medicine.

4.1 INTRODUCTION

Deoxyhypusine synthase (DHS) catalyzes the post-translational formation of the amino acid hypusine. Hypusine is unique to the eukaryotic translation initiation factor 5A (eIF5A), and is required for its functions in mRNA shuttling, translational elongation, and stress granule formation. In recent studies, we showed that DHS promotes acute cytokine and ER stress signaling in the islet β cell, thereby contributing to its dysfunction in the setting of diabetes mellitus (133,162). Because many of the previous studies of DHS involved small molecule inhibitors of the enzyme, such as GC7, I chose to investigate developmental and metabolic phenotypes associated with genetic DHS loss of function mutation, using conventional, whole body, DHS knockout (KO) mice.

Additionally, I investigated consequences of genetic DHS deficiency in *Dhps* knockout MEFs.

4.2 RESULTS

A. *Dhps* heterozygosity does not alter growth or metabolic homeostasis

Fig. 20A shows our *Dhps* gene targeting strategy. Exons 1-7 were replaced by a neomycin selection cassette, preserving exons 8 and 9 (exon 9 contains a gene, *Wdr83*, on the reverse strand). This strategy ensures that no elements containing the catalytic or binding domain of the DHS protein are produced. Using this targeting strategy, mice harboring a *Dhps* knockout allele (*Dhps*^{+/-}) were generated on a mixed *C57BL6/129SvEv* genetic background. Based on the analysis of offspring from 20 separate matings between *Dhps*^{+/-} mice (total of 99 offspring), I have obtained 34 *Dhps*^{+/+} mice, 65 *Dhps*^{+/-} mice, and no *Dhps*^{-/-} mice. Given the expected Mendelian ratio of 2:1 for *Dhps*^{+/-}:*Dhps*^{+/+} mice, and the absence of *Dhps*^{-/-} mice, I conclude that the *Dhps*^{-/-} genotype is embryonic lethal. I subsequently analyzed embryos from an additional 3 pregnancies at embryonic day 8.5-9.5, but was unable to identify any *Dhps*^{-/-} embryos. I next focused on the phenotype of *Dhps*^{+/-} mice. As shown in Fig. 20B-D, there is no difference between *Dhps*^{+/+} and *Dhps*^{+/-} mice with respect to body weight as mice age from 5-25 weeks, or in glucose homeostasis at 5 and 25 weeks of age (as assessed by intraperitoneal glucose tolerance tests (IPGTTs)).

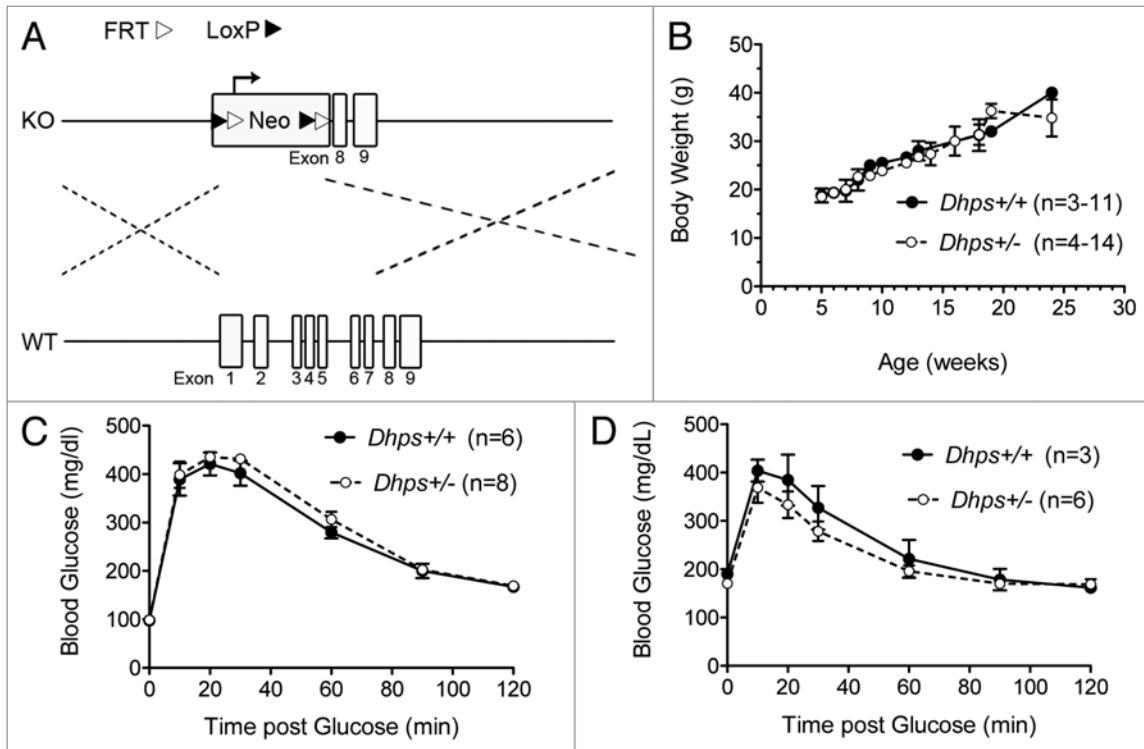


Figure 20: *Dhps* heterozygosity does not alter growth or metabolic homeostasis in mice. A, schematic diagram of the *Dhps* gene targeting vector (KO), and the wild type mouse locus (WT). Dotted lines represent homologous recombination regions. Neo, neomycin selection cassette; B, serial body weights of *Dhps*^{+/+} and *Dhps*^{+/-} mice between 5-25 weeks of age; C, results of glucose tolerance tests in mice at 5 weeks of age; D, results of glucose tolerance tests in mice at 25 weeks of age.

B. Dhps heterozygosity attenuates acute cytokine signaling in mouse embryonic fibroblasts

Although the growth and metabolic characteristics of *Dhps*^{+/-} mice appear unaffected, they do not rule out the possibility that reductions in DHS protein may still lead to phenotypic differences under conditions of stress. As an initial approach to address this possibility, I isolated mouse embryonic fibroblasts (MEFs) from *Dhps*^{+/+} and *Dhps*^{+/-} embryos at embryonic day 13.5. Compared to *Dhps*^{+/+} MEFs, *Dhps*^{+/-} MEFs contain approximately 50% less DHS protein as assessed by immunoblot analysis (Fig. 21A). The hypusine reaction can be monitored by polyacrylamide gel electrophoresis after incubating cells with the radiolabelled cofactor ³H-spermidine (see Chapter 2.2E). Although total eIF5A protein is unchanged between *Dhps*^{+/+} and *Dhps*^{+/-} MEFs, the rate of ³H-spermidine incorporation into eIF5A is reduced by approximately 40% in *Dhps*^{+/-} MEFs (Fig. 21B), a finding consistent at the protein level with haploinsufficiency of *Dhps* at the genetic level. To assess the response of haploinsufficient cells to the type of stress observed in diabetes, I subjected MEFs to incubation with a cocktail of proinflammatory cytokines (IL-1 β , TNF- α , and IFN- γ) for 4h. In prior studies, I showed that proinflammatory cytokines cause acute induction of the mRNA and protein for iNOS, and that inhibition of DHS with GC7 blocks expression of iNOS protein, but not its mRNA (133). As shown in Fig. 21C and D, activation of the mRNA encoding iNOS (*Nos2*) is unaffected in *Dhps*^{+/-} MEFs, but the production of iNOS protein is reduced by 60%. Although these data are not direct evidence of translational control of *Nos2* mRNA, the dissociation

between the mRNA levels and protein levels are nonetheless suggestive. These findings align with prior studies, and verify at the level of *Dhps* haploinsufficiency what we observed *in vitro* and *in vivo* with DHS inhibitors (133).

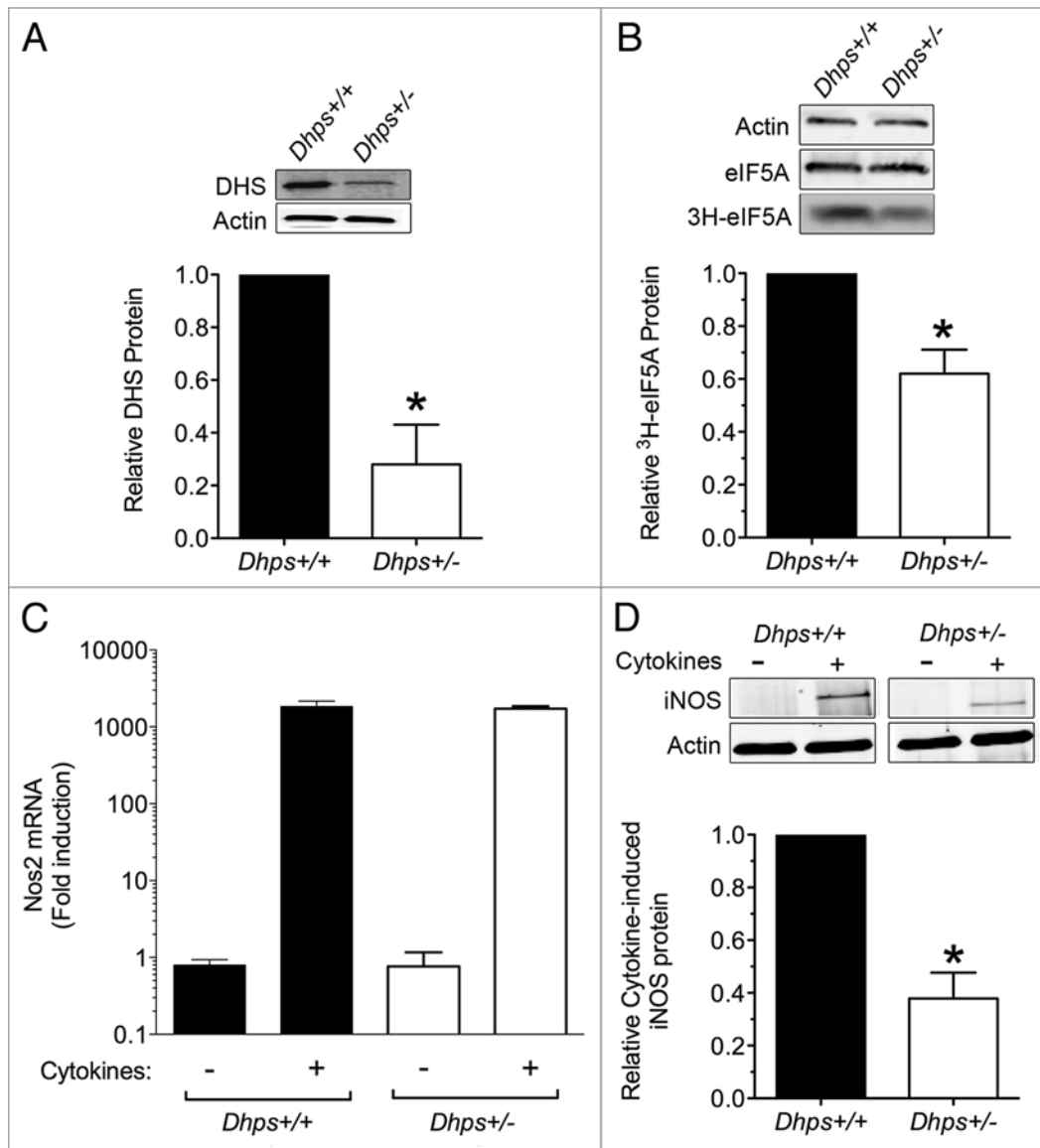


Figure 21: Dhps heterozygosity attenuates acute cytokine signaling in mouse embryonic fibroblasts. A, immunoblot analysis for DHS and actin from whole cell extracts of *Dhps*^{+/+} and *Dhps*^{+/-} MEFs (top panel), and quantification of DHS protein levels (bottom panel); B, MEFs were incubated with 1μCi ³H-spermidine for 4h, then subjected to immunoblot analysis of eIF5A and actin, or to fluorography for ³H-eIF5A following SDS-PAGE (top), and ³H-eIF5A was quantified (bottom); C, MEFs were incubated in the presence or absence of cytokines for 4h, then cDNA was subjected to qRT-PCR for Nos2; D, immunoblot analysis of iNOS and actin from whole cell lysates of MEFs treated with or without cytokines for 4h (top), and quantification of iNOS expression (bottom). Data represent mean ± SEM. n≥3. *P<0.05 compared to empty vector.

C. Dhps heterozygosity does not lead to significant inhibition of proliferation and G1/S cell cycle progression

To address the possibility that inhibition of DHS leads to defects in cellular proliferation, I performed ^3H -thymidine uptake studies in MEFs. As shown in Fig. 22A, *Dhps*^{+/-} MEFs demonstrated a roughly 40% lower ^3H -thymidine uptake over a 4h period compared to *Dhps*^{+/+} cells, although this difference did not strictly reach statistical significance ($p=0.06$). This reduction in cellular proliferation in *Dhps*^{+/-} cells correlated to a small, but again statistically insignificant, increase in the proportion of cells in G1 phase and a decrease in the proportion of cells in the S phase, a finding consistent with trend to G1/S block (Fig. 22B). By contrast, under cytokine stress conditions, no obvious differences in proliferation or cell cycle populations were observed between the two genotypes (Fig. 22C and D). Overall, however, the lack of statistically significant changes in cell cycle populations is consistent with the observation that *Dhps*^{+/-} mice displayed no obvious differences in growth or weight up to 6 months of age.

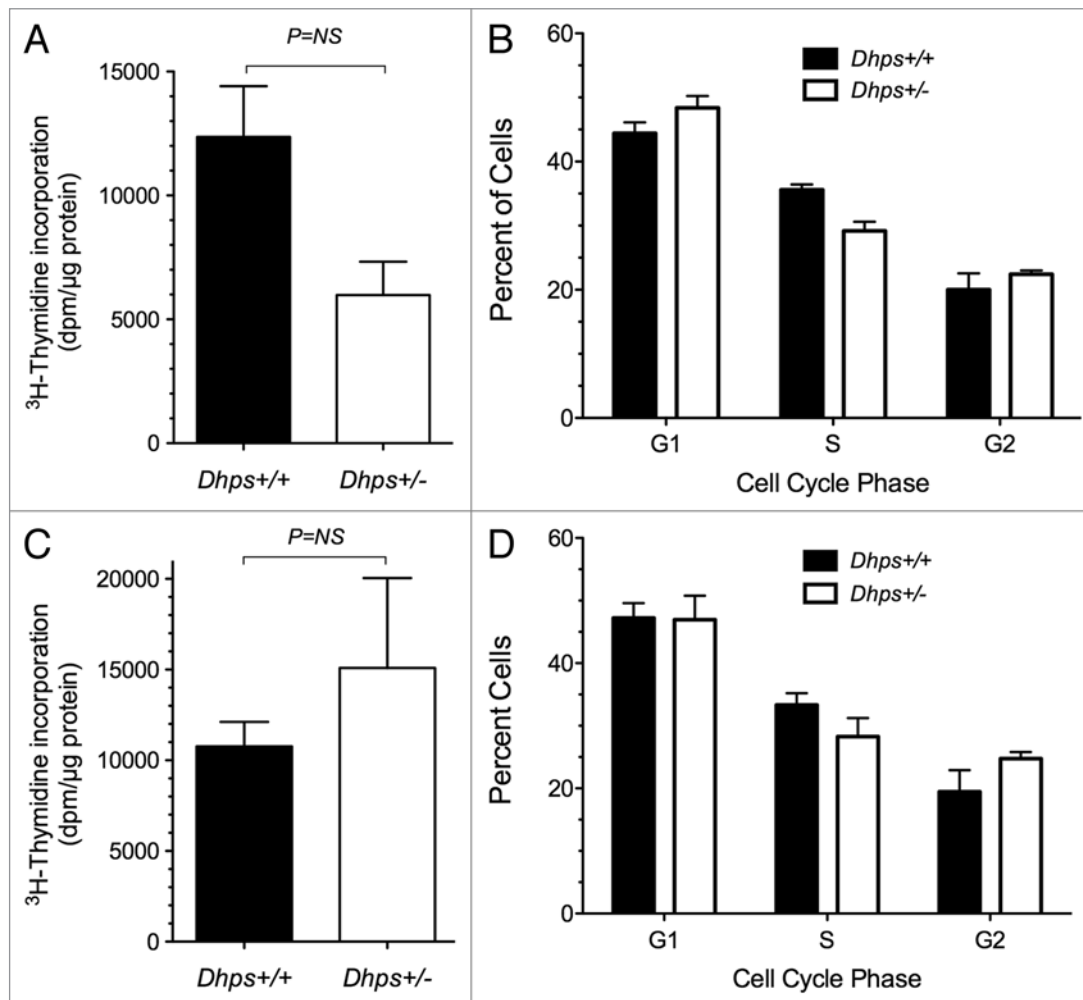


Figure 22: Dhps heterozygosity does not lead to significant inhibition of proliferation and G1/S cell cycle progression. A, MEFs were incubated with $1\mu\text{Ci}$ ^3H -methyl thymidine for 4h, then washed and lysed. ^3H -thymidine incorporation was measured and normalized to protein content; B, MEFs were incubated with Guava cell cycle reagent for 30 min, and intercalation of propidium iodide into cellular DNA was quantified using FACS; C, same as in A, except cells were treated concurrently with cytokines and ^3H -methyl thymidine for 4h; D, same as B, except cells were treated with cytokines for 4h prior to incubation with Guava reagent. Data represent mean \pm SEM. $n\geq 3$. * $P<0.05$ compared to empty vector.

4.3 DISCUSSION

Prior studies have pointed to a potential role for DHS and hypusinated eIF5A in the responses of islet β cells to proinflammatory and ER stressors. Given the totality of the data on the two proteins, it is fair to ask how precisely this pair should be viewed with respect to cellular survival. On one hand, depletion of eIF5A or inhibition of DHS appears to be beneficial, preventing hyperglycemia in mouse models of inflammation-induced diabetes (133,162); in another particularly striking study, depletion of eIF5A protects mice against death in the setting of sepsis induced by lipopolysaccharide (187). On the other hand, inhibition of DHS or depletion of eIF5A can also have negative effects, leading to defects in both cell cycle progression and the formation of stress granules (159). Further, I show that *Dhps*^{-/-} mice are early embryonic lethal. Thus, it is possible that DHS is important either in the proliferation of early embryonic cells and/or in the ability of those cells to activate stress signaling pathways during differentiation.

With respect to the islet β cell, it may be best to view DHS and eIF5A as representing stress-responsive proteins whose functions are accentuated in periods of acute stress (such as inflammation, viral infection, sepsis, etc.). Under these stress conditions, I propose that DHS and eIF5A are necessary for regulating the ultimate translation of proteins that are involved in stress remediation or adaptation; however, as the stress continues unabated, these proteins may be required to trigger the cellular execution response in an attempt to limit the extent of stress. Thus, DHS and eIF5A may regulate the balance

between adaptive and pro-death responses in the cell. As such, it is possible that too much or too little of these proteins may shift the balance in favor of cellular death. The *db/db* mouse model may represent a particularly dramatic example of this balance in the islet: the *C57BL/6J-db/db* strain attenuates eIF5A hypusination rates sufficiently to favor islet adaptation and normoglycemia, whereas the *C57BKLS/J-db/db* strain maintains hypusination rates that favor islet death (162).

Hypusination may be an attractive therapeutic target in the islet β cell for several reasons. First, hypusinated eIF5A has a very short half-life in islets (~6 h) compared to other cell types (>20 h) (133,188–190), a finding supporting an acute regulatory role for the protein in islets. Also, eIF5A and DHS exhibit rapid and reciprocal nucleo-cytoplasmic shuttling in β cells in response to cytokines or ER stress (133,162), whereas in other cell types specific compartmentalization is less clear or controversial (168,191). Finally, the islet β cell has a very slow replicative rate and therefore is less susceptible acutely to agents (such as DHS inhibitors) that affect cellular proliferation.

Our data suggest that *Dhps*^{+/-} mice can maintain normal growth and glucose homeostasis (Fig. 20), and that their cells attenuate responses to inflammation. Indeed, *Dhps*^{+/-} MEFs exhibit reduced iNOS protein expression in response to cytokine exposure compared to wild type controls (Fig. 21), a result that supports our findings using the pharmacological DHS inhibitor GC7. In these respects, I believe pharmacologic approaches to inhibiting DHS may be successful in mitigating diabetes progression in animal models. Nonetheless,

further research using islet and cell-specific knockout mouse models of DHS and eIF5A will be especially helpful in elucidating the roles of these proteins in islet cell types and diabetes models.

Recent work has suggested that hypusinated eIF5A functions to alleviate ribosome stalling at specific amino acid sequences, including polyproline motifs (138,139). Therefore, DHS and eIF5A may be necessary for efficient translation of a subset of transcripts within the cell, and eIF5A hypusination may represent an important mechanism of translational regulation. Although studies of the function of hypusinated eIF5A provide interesting insight into the precise mechanism of eIF5A, as well as potential targets, further studies are required to reveal the physiological significance of this system with regards to diabetes.

CHAPTER 5

Translational Control of Inducible Nitric Oxide Synthase by p38 MAPK in Islet β Cells

Given the role of deoxyhypusine synthase in translational of specific mRNAs, we next investigated how DHS activity is regulated. In this chapter, I explore the role of MAPKs in translational regulation of iNOS, and suggest a role for p38 signaling in deoxyhypusine synthase activation in the β cell. Mechanisms of DHS and p38 facilitated iNOS translation are explored. Immunoblot and fluorography studies were done in collaboration with Dr. Yurika Nishiki at Indiana University School of Medicine.

5.1 INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are transducers of extracellular signals, such as proinflammatory cytokines. In islet β cells, cytokines acutely activate expression of the *Nos2* gene encoding inducible nitric oxide synthase (iNOS), which ultimately impairs insulin release. Because *Nos2* can also be regulated post-transcriptionally, we asked if MAPKs participate in events following *Nos2* transcription in β cells and primary islets. DHS and eIF5A are believed to be necessary for translation of a specific subset of β cell stress responsive transcripts, including *Nos2*. However, relatively little is known about how expression of hypusinated eIF5A is regulated in response to stress in the β cell, and how this affects translation. Therefore, we undertook experiments to

explore the relationship between MAPK signaling, DHS activity, and iNOS protein expression.

5.2 RESULTS

A. Inhibition of p38 partially blocks iNOS protein production.

Prior studies have shown that p38 inhibition partially blocks activation of the gene encoding iNOS (*Nos2*) within the 24h timeframe following cytokine exposure (192). Fig. 23A and B show that in the timeframe of our studies (4h), there was no significant effect of p38 inhibition by PD169316 on the activation of *Nos2* in INS-1 β cells or rat islets. The same result was true of inhibition of the MAPK c-Jun N-terminal kinase (JNK) by SP006125 (Fig. 23A and B). Next we examined the effect of p38 inhibition by PD169316 on cytokine-induced iNOS protein levels by immunoblot. As shown in Fig. 23C and D, p38 inhibition attenuated cytokine-induced iNOS protein expression by ~40% in INS-1 cells and by ~50% in rat islets compared to controls. By contrast, inhibition of the MAPK JNK with SP600125 did not affect iNOS protein levels (Fig. 23C and D). Whereas cytokines enhanced the phosphorylation of p38 in INS-1 cells, inhibition of p38 blocked its own phosphorylation (Fig. 23C)—a finding consistent with known effects of p38 inhibitors. The results in Fig. 23 suggest that p38 activity enables a post-transcriptional process that maintains normal iNOS protein levels, reminiscent of the role of DHS in iNOS translation.

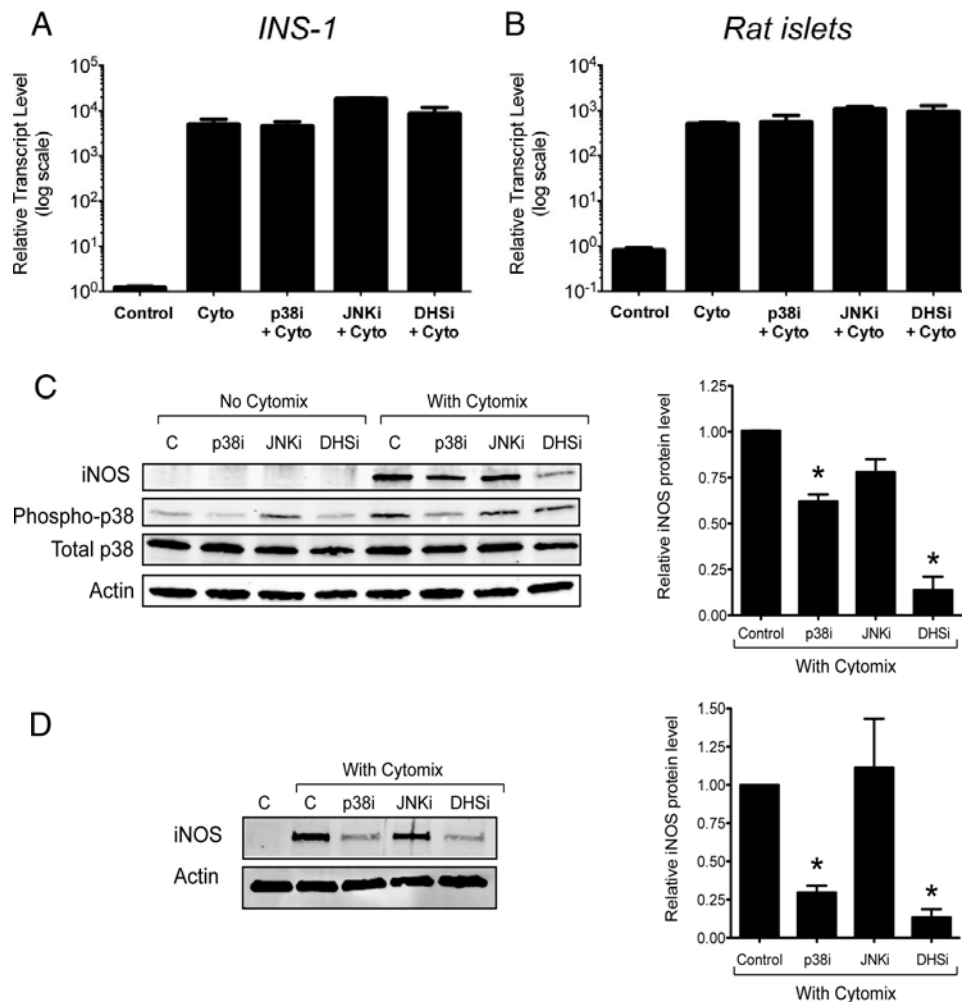


Figure 23: Effect of enzyme inhibitors on cytokine-induced *Nos2* mRNA and iNOS protein expression. A, INS-1 rat β cells, and B, rat islets were untreated, or treated with cytokines for 4h in the presence or absence of the indicated inhibitors, cells were harvested and cDNA was subjected to qRT-PCR for *Nos2* message. Data corrected for *Actb* mRNA, and compared to untreated control; C, INS-1 β cells, and D, rat islets were untreated, or treated with cytokines for 4h in the presence or absence of the indicated inhibitors, then whole cell lysates were subjected to immunoblot analysis of protein expression. Data corrected for actin expression and quantified. Data represent mean \pm SEM. $n=3$. * $P<0.05$ compared to untreated control (A, B), or cytokine-only control (C, D).

B. p38 and DHS activity promote translational elongation of *Nos2* mRNA

The data in Fig. 23 suggest that p38 activity contributes to translation of *Nos2* mRNA. To interrogate more directly the possibility that p38 activity is required for *Nos2* translation, I performed polyribosome profile (PRP) analysis using INS-1 β cells. INS-1 cells were incubated with cytokines in the absence or presence of inhibitors of translational initiation (thapsigargin, Tg), translational elongation (cycloheximide, CHX), p38 (PD169316), JNK (SP600125), and DHS (GC7). Fig. 24A shows the INS-1 cell PRP after 4h of cytokine incubation, identifying the positions of the 40S, 60S, 80S ribosome-associated RNA, as well as the position of polyribosome associated RNA. Co-incubation with cytokines and Tg resulted in a dramatic enhancement to monoribosome- and diribosome-associated RNA and near-complete dissipation of polyribosome-associated RNA (Fig. 24B), consistent with a block of translation initiation. Importantly, the *Nos2* mRNA, which is associated primarily with actively translating polyribosomes under cytokines conditions, shifts leftward toward the monoribosome-associated fractions in the presence of Tg (Fig. 24B, shaded region), indicative of translation initiation blockade of *Nos2*. By contrast, incubation with CHX leads to increases in both monoribosome- and polyribosome-associated RNAs, findings that reflecting a block in translational elongation (Fig. 24C). In this case, *Nos2* mRNA is retained in the polyribosome fractions. When INS-1 cells are treated with p38 and JNK inhibitors, there remains retention of polyribosome-associated RNAs, comparable to that seen with CHX (Fig. 24D and E). Treatment with the DHS inhibitor GC7 results in a PRP similar to the MAPK inhibitors, but with greater

monoribosome and diribosome-associated RNAs (Fig. 24F). Similar to CHX treatment, the *Nos2* mRNA remains largely associated with polyribosomes in the setting of MAPK and DHS inhibition (Fig. 24D-F, shaded region), suggesting that this mRNA is translationally blocked at the elongation phase.

To clarify whether p38 promotes translational elongation specifically in the setting of cytokine stress, I next performed PRP studies using *p38α* *+/+* and *p38α* *-/-* MEFs. As shown in Fig. 25A, comparison of the PRP of *p38α* *+/+* MEFs to that of *p38α* *-/-* MEFs revealed an accumulation of polyribosome associated RNAs in the *p38α* *-/-* MEFs, suggestive of a translation elongation blockade. After *p38α* *+/+* MEFs were incubated with Tg for 4h (Fig. 25B), there is prominence of the 80S peak (reflective of a translation initiation block caused by Tg). In contrast to *p38α* *+/+* cells, *p38α* *-/-* cells exhibit greater retention of polyribosome-associated RNAs upon treatment with Tg (Fig. 25B, right panel), again suggesting that the absence of p38α results in a partial defect in translational elongation. Thus, I conclude that p38 promotes translational elongation, and suggest a link between p38 and hypusinated eIF5A.

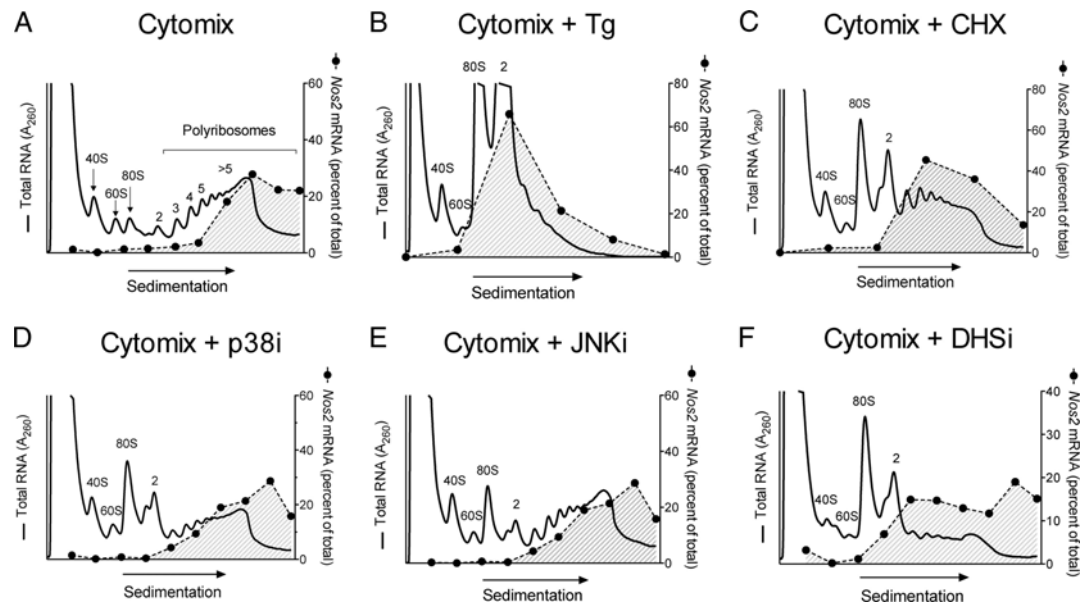


Figure 24: Effect of enzyme inhibitors on *Nos2* mRNA ribosomal occupancy. INS-1 β cells were cultured with cytokines for 4h in the presence or absence of the indicated inhibitors, then subjected to PRP analysis with fractionation of the sedimentation gradient, and analysis of *Nos2* mRNA in PRP fractions. Cells were treated with A, cytokines alone, B, cytokines plus thapsigargin, C, cytokines plus CHX, D, cytokines plus p38 inhibitor, E, cytokines plus JNK inhibitor, F, cytokines plus DHS inhibitor. Closed circles encompassing shaded areas illustrate the distribution of *Nos2* mRNA. Data shown are representative profiles obtained from 3-4 independent experiments.

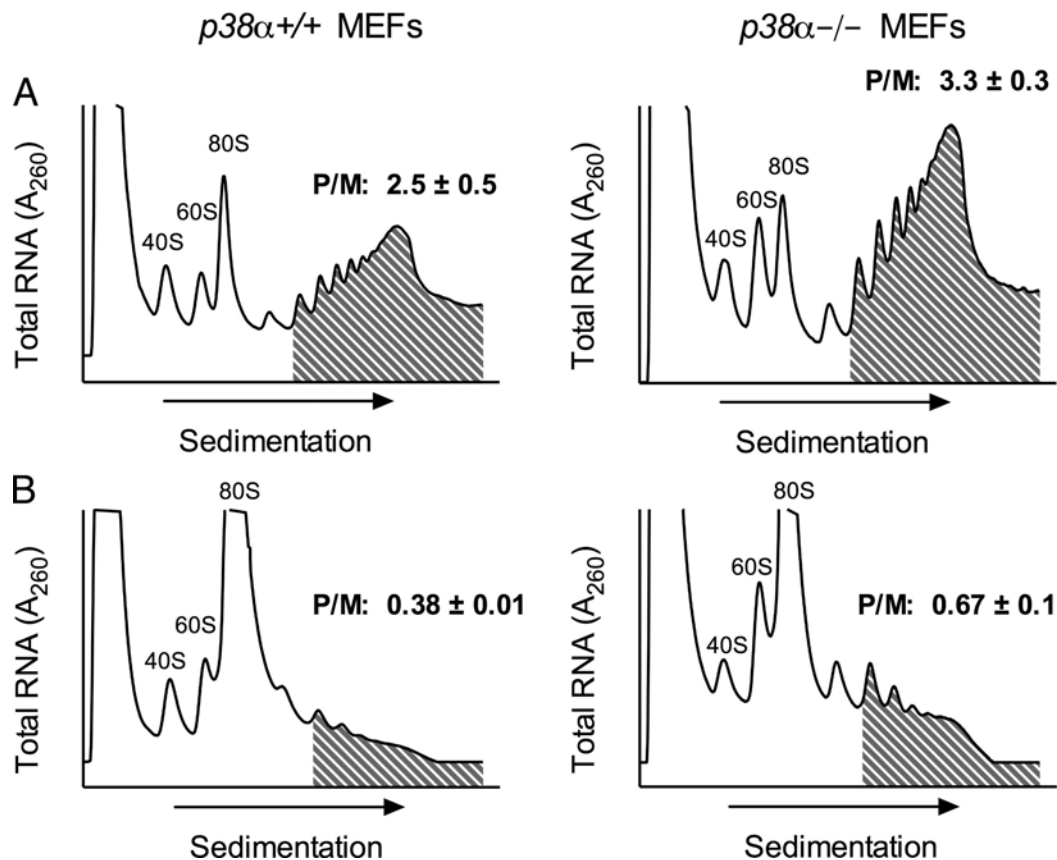


Figure 25: p38 activity promotes translational elongation. A, *p38*^{+/+} MEFs (left panel) and *p38*^{-/-} MEFs (right panel) were treated with cytokines for 4h, and then subjected to PRP; B, *p38*^{+/+} MEFs (left panel) and *p38*^{-/-} MEFs (right panel) were treated with cytokines plus 1 μ M Tg for 4h, and then subjected to PRP. Shaded area indicates polyribosome region. P/M ratio values are shown for each profile.

C. p38 activity contributes to eIF5A hypusination

These results point to a role for p38 activity in *Nos2* translational elongation that appears similar to that of DHS, an enzyme that is required for the function of eIF5A (133). We therefore asked whether the apparent translational effect of p38 might be related directly to the function of eIF5A. The translational elongation function of eIF5A is dependent upon the hypusine modification of Lys50, a reaction that is sequentially catalyzed by DHS and deoxyhypusine hydroxylase (164). DHS activity can be measured directly by ³H-spermidine incorporation into eIF5A (see Chapter 2.2E). As anticipated, INS-1 β cells, rat islets, and human islets treated with the DHS inhibitor GC7 exhibited a disruption in eIF5A hypusination (Fig. 26A-C) (193). Whereas the JNK inhibitor SP600125 had no significant effect on hypusine incorporation, the p38 inhibitor PD169316 caused a significant decrease in hypusination in both the β cell line and primary rat and human islets in the presence of cytokines (Fig. 26A-C). Interestingly, no effect of p38 inhibition on hypusine incorporation was observed in the absence of cytokines, implying an effect of p38 that is cytokine dependent. Taken together, these data indicate that p38 MAPK promotes activity of DHS in the presence of cytokine signaling, leading to enhanced translational elongation of *Nos2* mRNA.

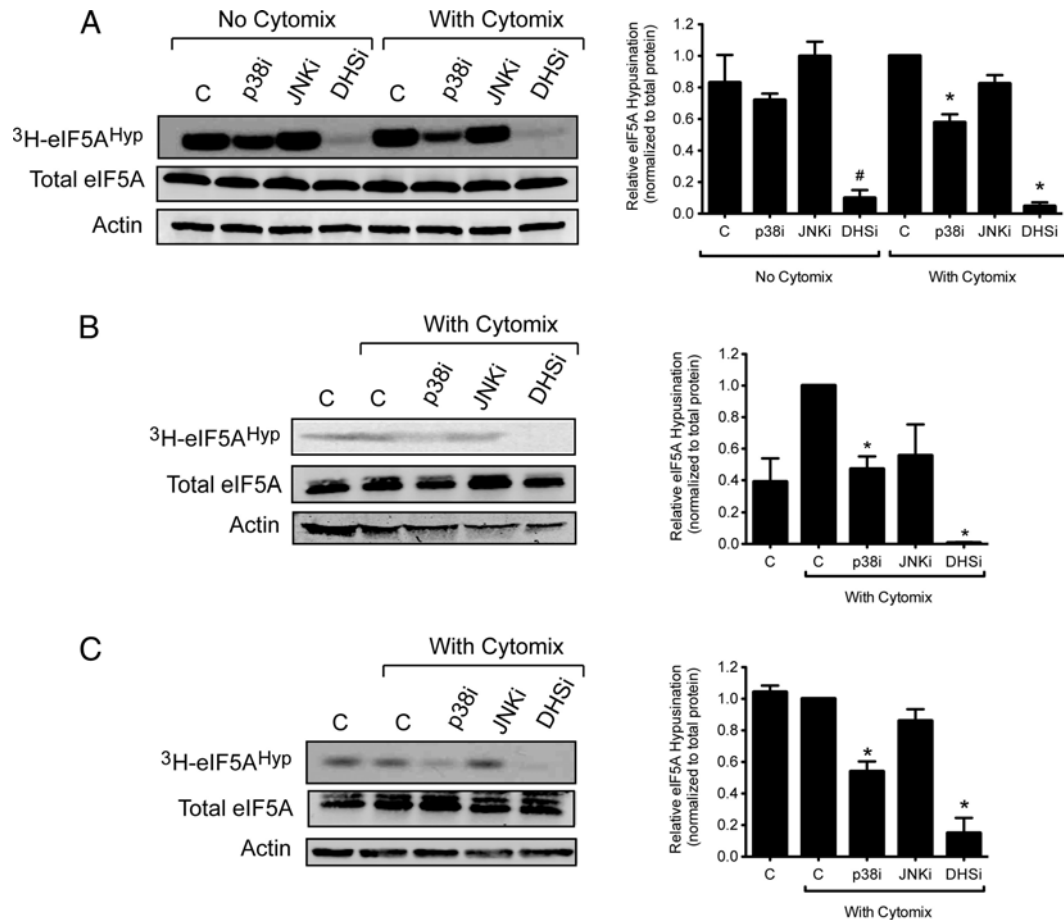


Figure 26: Effect of enzyme inhibitors on eIF5A hypusination. A, INS-1 β cells, B, rat islets, or C, human islets were exposed to ^3H -spermidine for 4h in the presence or absence of cytokines and the indicated inhibitors, then whole cell extracts were harvested and subjected to SDS-PAGE and fluorography for hypusinated eIF5A (^3H -eIF5A^{Hyp}) or immunoblotting for total eIF5A and actin. Panels on the left show representative fluorography and immunoblots, while panels on the right show quantification of hypusinated eIF5A normalized to actin. Data represent mean \pm SEM. $n=3$. $^*P<0.05$ compared to cytokine only control.

5.3 DISCUSSION

Proinflammatory cytokines have been implicated in the pathogenesis of islet β cell dysfunction in both type 1 and type 2 diabetes mellitus, and potential therapies targeted against cytokines or their signaling pathways have been proposed as approaches to preserve β cell function and survival in both diseases. The MAPKs have been suggested as therapeutic targets in the setting of diabetes because their actions downstream of cytokine signaling promote both transcription and translation of mRNAs that contribute to the dysfunction or death of a variety of cell types that regulate metabolic homeostasis (for review, see ref. (194)). Our studies suggest a novel mechanism whereby MAPKs, specifically p38, support the translational elongation of *Nos2* mRNA via hypusination of the translational factor eIF5A.

Polyribosomal profiling experiments using INS-1 β cells revealed that *Nos2* mRNA remained associated with polyribosomes in the setting of p38 inhibition, in a manner similar to that seen with blockade of translation elongation. Consistent with a role in translation elongation, p38 activity contributes to hypusination of eIF5A. Our results suggest a novel signaling pathway in which p38 MAPK promotes translation elongation of *Nos2* mRNA via regulation of eIF5A hypusination.

Translational elongation at the ribosome is dependent upon the interplay of a group of translational factors that are distinct from those involved in the initiation of translation (reviewed in ref. (150)). Interestingly, the translation factor eIF5A was originally identified as an initiation factor based on early studies of in

vitro methionyl-puromycin synthesis (195). In the intervening years, its role as an initiation factor has been questioned and more recent studies suggest it functions primarily as an elongation factor (139,158). Nevertheless, controversy still exists in the literature as to whether eIF5A functions primarily as an initiation or elongation factor (196).

The post-translational hypusine modification of eIF5A is required for the known translational and RNA binding functions of eIF5A (164). In recent studies we demonstrated that hypusinated eIF5A is required for nuclear export and translation of *Nos2* mRNA in β cells (133). Here, we show that p38 inhibition leads to reduced hypusination in the setting of cytokine signaling, resulting in reductions in iNOS protein that parallel effects observed with inhibition of DHS. Although both DHS and DOHH contain putative MAPK phosphorylation sites, I should note that our data do not necessarily imply that p38 directly modifies (phosphorylates) either protein. Studies currently ongoing will evaluate the possibility that direct phosphorylation of DHS by p38 may be necessary for the activity and/or stability of DHS.

Taken together, our data suggest a pathway whereby p38 activity links, in part, the transduction of cytokine signals with activation of hypusination. Although I cannot rule out the possibility that other translational elongation factors, such as eEF2, also participate in the translational elongation of *Nos2* promoted by p38, it is possible that these factors are all linked closely to one another such that loss in the activity of one can affect the assembly of the full translational elongation complex. In the case of eIF5A, the factor does not appear to function as a

generalized translational factor, but instead promotes the elongation of only a subset of mRNAs in the β cell (133,158,159). Thus, our observations in this study may represent a novel p38-linked translational pathway for specific stress responsive transcripts. A limitation in these studies is the use of small molecule inhibitors, although the concern for off-target effects is mitigated by our parallel findings in *p38 α* $-/-$ MEFs. Nevertheless, because such inhibitors may find their way into the clinical settings of type 1 and type 2 diabetes, our studies provide new insight into the mechanisms by which MAPK inhibition might limit the consequences of inflammation on the islet β cell.

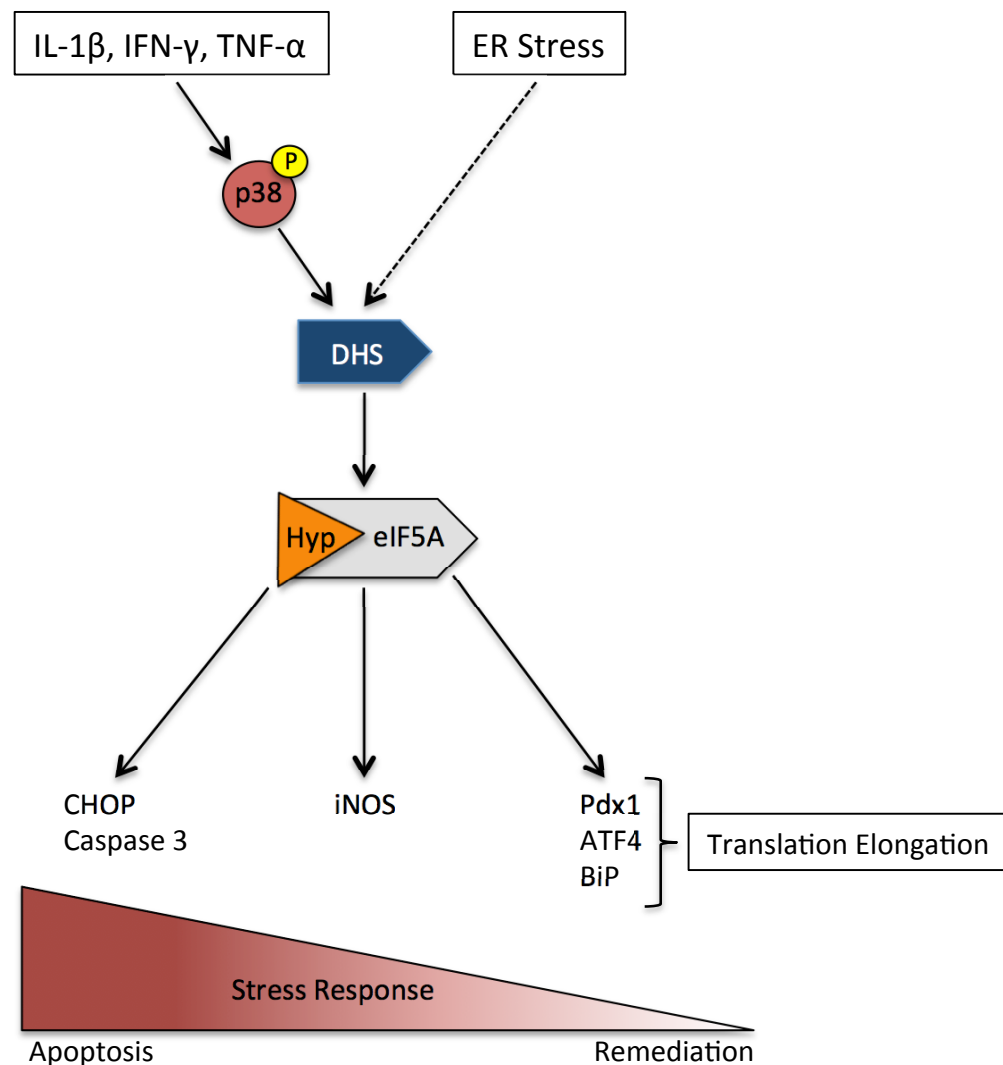


Figure 27: Role of DHS and eIF5A in the β cell stress response. DHS and eIF5A are interrelated proteins necessary for efficient translational elongation of a number of β cell transcripts. Current reports indicate hypusine-eIF5A is involved in translational elongation of both stress remediating and death inducing factors (133,162). Therefore, the cellular balance of hypusinated eIF5A may be key in determining β cell fate.

CHAPTER 6

Conclusions

Failure of the islet β cell is now recognized as the major event leading to onset of diabetes (2). Thus, a greater understanding of β cell dysfunction and death is critical to devising new treatments for preservation of β cell function, and to preventing diabetes. Whereas β cell transcriptional responses to stress are well understood, less is known about β cell translational responses, and how these may mitigate, or contribute to β cell dysfunction.

6.1 REGULATION OF β CELL TRANSLATION INITIATION

In the studies outlined here, I have investigated translational stress responses of the pancreatic β cell. First, I determined that chronic treatment of β cells with proinflammatory cytokines or palmitate results in loss of polyribosome associated RNAs, consistent with attenuation of translation initiation, which is a feature of ER stress. This loss of polyribosome associated RNA coincided with decreased total protein synthesis, further suggesting ER stress in these models. In addition to changes in general translation status, I observed transcript specific regulation of ribosomal occupancy in β cells. *Atf4* and *Chop* are translationally activated by proinflammatory cytokine or Tg treatment in the β cell, whereas *Cpe* is translationally repressed. Interestingly, ribosomal occupancy of *Pdx1* mRNA did not change under any of the conditions I studied, indicating it is translationally regulated by a distinct mechanism. Further studies identified a regulatory region

in the *Pdx1* 5' UTR that allows maintenance of translation initiation in the setting of the UPR.

Although more studies are required to fully understand the mechanism of stress resistant Pdx1 translation, my experiments identify the region from nucleotides -105 to -280 of the Pdx1 5' UTR as a positive regulator of translation initiation. A better understanding of the factors allowing operation of this mechanism may shed light into causes of decreased Pdx1 expression in diabetes, or alternatively, provide new ways to enhance Pdx1 expression in the islet β cell, thereby improving β cell function. Therefore, a greater understanding of *Pdx1* translation initiation will lead to therapies designed to promote Pdx1 expression, thus maintaining β cell identity and function, and preventing diabetes.

6.2 REGULATION OF β CELL TRANSLATION ELONGATION

In addition to studying mechanisms of translation initiation in the β cell, I also studied the roles of DHS and eIF5A in β cell translational elongation. Initial experiments with whole body *Dhps* knockout mice demonstrated that the *Dhps* $-/-$ genotype is embryonic lethal before day E9.5. In contrast, *Dhps* $+/-$ mice appeared metabolically healthy, with normal weight and glucose tolerance between 5 and 25 weeks of age. Additionally, MEFs isolated from *Dhps* $+/-$ mice displayed reduced DHS protein expression and activity, and attenuated cytokine induced iNOS expression compared to wild type MEFs. This reduction in iNOS expression is mediated post-transcriptionally.

These data provide insight into the role of DHS in development, and support at the genetic level experiments carried out using small molecule DHS inhibitors. Specifically, these experiments demonstrate the importance of DHS in cytokine induced *Nos2* translation. Given the hypothesized role of DHS in allowing translation of specific stress responsive mRNAs, DHS inhibition may represent a inhibition may representation Indeed, DHS inhibition results in reduced *Nos2* translation and improved glucose tolerance in mouse models of diabetes (165). However, a greater knowledge of the translational targets of hypusinated eIF5A is needed to fully grasp the physiological outcomes of β cell DHS inhibition. Ongoing studies of pancreas and β cell specific *Dhps* knockout mice will be critical to our understanding of the role of DHS in β cell translational responses.

In addition to investigating targets of DHS and eIF5A, I was also interested in understanding how DHS activity is regulated in response to stress. In this regard, we investigated the roles of the MAPKs p38 and JNK in eIF5A hypusination and cytokine induced iNOS expression. Similar to DHS inhibition, we determined that p38 inhibition resulted in attenuated cytokine induced iNOS expression, and that this reduction was not due to differences in *Nos2* mRNA abundance. Next, using PRP, I demonstrated that both DHS inhibition and p38 inhibition result in an apparent block of *Nos2* translational elongation. Interestingly, p38 $-/-$ MEFs displayed increases in polyribosome associated RNAs, also suggestive of a role for p38 in translational elongation. Finally, we

demonstrated that inhibition of p38 results in decreased DHS activity, as assessed by ³H-spermidine incorporation.

These data suggest a role for p38 in translational elongation of *Nos2* mRNA, possibly via induction of DHS activity. As such, p38 inhibition may represent another therapeutic strategy for maintenance of β cell function. Although the effects of p38 inhibition on *Nos2* mRNA translation are similar to those of DHS inhibition, our studies do not necessarily imply a direct activation (phosphorylation) of DHS by p38. Future studies exploring direct phosphorylation of DHS by p38 will add significantly to knowledge of stress induced regulation of DHS activity.

6.3 FINAL THOUGHTS

Islet β cell research has demonstrated that loss of β cell function is the key determinant in development of both type 1 and type 2 diabetes (42,59). β cell translational regulation, which is a consequence of inflammation, ER stress, and the UPR, represents a novel area of research for development of therapeutics targeted at improving β cell function. Translational regulation in the islet β cell is an important determinant in continued β cell function and health (129,197). Thus, continued research into β cell translation regulatory mechanisms are vital for addressing the problem of β cell dysfunction in diabetes. Ultimately, exploitation of mechanisms of β cell translational regulation including 5' UTR mediated *Pdx1* translation initiation and DHS facilitated *Nos2* translation elongation will lead to therapeutics for improved β cell function and survival.

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Indiana University School of Medicine, Indianapolis, IN (2009 – Present)*
Advisor: Raghu Mirmira, MD, PhD
Investigating the role of deoxyhypusine synthase (DHS) in stress induced regulation of inflammatory transcript shuttling and translation in islet cells. Studies incorporate DHS knock out mice, isolated islets, and mouse embryonic fibroblasts.

*Division of Pulmonary and Critical Care Medicine, Research Technologist
Northwestern University Feinberg School of Medicine, Chicago, IL (2007 – 2009)*
Advisor: Karen Ridge, PhD
Used cell and molecular biology techniques to investigate regulatory mechanisms of intermediate filament organization in alveolar epithelial cells.

OTHER RELEVANT EXPERIENCE

*Department of Biochemistry and Molecular Biology, Rotating Student
Indiana University School of Medicine, Indianapolis, IN (January – March 2010)*
Advisor: Peter Roach, PhD
Studied the role of genethonin in lysosomal glycogen storage, and constructed point mutated genethonin to investigate the potential regulation of genethonin by AMPK.

*Department of Cellular & Integrative Physiology, Rotating Student
Indiana University School of Medicine, Indianapolis, IN (March –
April 2010)*

Advisor: Michael Sturek, PhD

Explored the effects of aminoimidazole carboxamide ribonucleotide (AICAR) on consequences of coronary ischemia in Ossabaw swine.

*Department of Health and Kinesiology, Volunteer Laboratory Assistant
Purdue University College of Liberal Arts, West Lafayette, IN (2000 –
2002)*

Advisor: Michael Flynn, PhD

Investigated the influence of resistance exercise on toll-like receptor 4 transcription and other markers of inflammation.

ACADEMIC HONORS

Indiana University School of Medicine (2009-Present)

Moenkhaus Physiology Graduate Fellowship

American Heart Association Pre-Doctoral Fellowship

Indiana University (2002-2006)

Dean's List

Golden Key International Honor Society

AED Pre-Medical Honor Society

Sigma Chi Fraternity scholarship recipient

GRANTS

Principle Investigator, "The Role of Deoxyhypusine Synthase in Pancreatic β -Cell Stress Response," American Heart Association, Midwest Affiliate, \$52,000, 7/1/2011 – 6/30/2013.

RESEARCH PUBLICATIONS

- Liew CW, Assmann A, **Templin AT**, Raum JC, Lipson KL, Rajan S, Hu J, Kawamori D, Lindberg I, Philipson L, Sonenberg N, Goldfine AB, Stoffers DA, Mirmira RG, Urano F, Kulkarni RN. Insulin Regulates Carboxypeptidase E by Modulating Translation Initiation Scaffolding Protein eIF4G1 in Pancreatic islet. Proceedings of the National Academy of Sciences. In Press.
- Hatanaka M, Maier B, Sims EK, **Templin AT**, Kulkarni RN, Evans-Molina C, Mirmira RG. Saturated Free Fatty Acids Induce mRNA Translation and Increase ER Protein Load in Islet β Cells. American Heart Association, Midwest Affiliate. Rapamycin Pathway. Diabetes. In Press.
- Nishiki Y, Adewola A, Hatanaka M, **Templin AT**, Maier B, Mirmira RG. Translational Control of Inducible Nitric Oxide Synthase by p38 MAPK in Islet β Cells. Molecular Endocrinology. 27(2):336-49 (2013)

- Tersey SA, Nishiki Y, **Templin AT**, Cabrera SM, Stull ND, Colvin SC, Evans-Molina C, Rickus JL, Maier B, Mirmira RG. Islet , Mirmiraairmiravin SC, Evans-Molina C, de Synthase by p38 MAPK in Islet d in IsleNonobese Diabetic Mouse Model. Diabetes. 61(4):818-27 (2012)
- **Templin AT**, Maier B, Nishiki Y, Tersey SA, and Mirmira RG. Deoxyhypusine Haploinsufficiency Attenuates Acute Cytokine Signaling. Cell Cycle. 10(7):1043-1049 (2011)

MANUSCRIPTS IN REVISION

- **Templin AT**, Maier B, Hatanaka M, Mirmira RG. Maintenance of Pdx1 mRNA Translation in Islet β Cells During the Unfolded Protein Response. Molecular Endocrinology. Under revision.

CONFERENCES

American Diabetes Association (2013)
Indiana Physiological Society (2011, 2013)
Midwest Islet Club (2010, 2011, 2012, 2013)

ORAL PRESENTATIONS

Midwest Islet Club – 2013 – Proinflammatory cytokines induce an ER stress response that activates an alternative mRNA translational program

Indiana Physiological Society – 2013 - Proinflammatory cytokines induce ER stress with concomitant alterations in mRNA ribosomal occupancy in islet β cells

POSTERS

American Diabetes Association – 2013 – Proinflammatory cytokines induce ER stress with concomitant alterations in mRNA ribosomal occupancy in islet β cells

Midwest Islet Club – 2012 – Proinflammatory cytokines cause mRNA translation blockade in islet β cells

Midwest Islet Club – 2011 – Deoxyhypusine synthase haploinsufficiency attenuates acute cytokine signaling while preserving growth and glucose homeostasis

PROFESSIONAL AFFILIATIONS

American Physiological Society – Student Member
American Association for the Advancement of Science – Sponsored Member

TEACHING EXPERIENCE

F503 – Human Physiology – Fall 2013 - Guest Lecturer

F716 – Molecular Biology – Fall 2013 – Discussion Facilitator

C750 – College Teaching and Learning – Fall 2011 – Student/Lecturer

OTHER

NIH Regional Seminar Volunteer – Indianapolis, Indiana (Spring 2012)

Molecular Medicine In Action – Indiana University (Fall 2010, 2011)

Indiana Physiological Society Study Section – Student Member

Interests: Alpine skiing, baseball, current events, reading